The Human Cell as an Environment for Horizontal Gene Transfer

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ABSTRACT

Horizontal gene transfer (HGT) is now indisputably the predominant driving force, if not the sole force, behind speciation and the evolution of novelty in bacteria. Of all mechanisms of horizontal gene transfer (HGT), conjugation, the contact-dependent plasmid-mediated transfer of DNA from a bacterial donor to a recipient cell, is probably the most universal. First observed between bacteria, conjugation also mediates gene transfer from bacteria to yeast, plant and even animal cells. The range of environments in which bacteria naturally exchange DNA has not been extensively explored. The interior of the animal cell represents a novel and potentially medically relevant environment for gene transfer. Since most antibiotics are ineffective inside mammalian cells, our cells may be a niche for the evolution of resistance and virulence in invasive pathogens. Invading bacteria accumulate in vacuoles inside human cells, protected from antibiotics. Herein, I demonstrate the ability of intracellular *Salmonella typhimurium* to meet and exchange plasmid DNA by conjugation within animal cells, revealing the animal intracellular milieu as a permissive environment for gene exchange. This finding evokes a model for the simultaneous dissemination of virulence and antibiotic resistance within a niche protected from both antibiotics and the immune system and extends the variety of environments in which bacteria are known to exchange genes.

Unlike conjugation between bacteria, conjugation between bacteria and eukaryotic cells requires the import of transferred DNA into the nucleus before the transferred genes can be expressed and inherited. Plant-cell nuclear transformation by the conjugation system of the *Agrobacterium tumefaciens* Ti plasmid is believed to be mediated by nuclear localization sequences (NLSs) carried within the proteins that accompany the T-DNA during transfer. Whether NLSs are equally important for transmission of other conjugative plasmids to eukaryotic cells is unknown. Herein, I demonstrate nuclear localization potential within the putative conjugative escort protein TraI of the IncPα plasmid RP4. In contrast, MobA, the putative escort protein from the IncQ plasmid RSF1010, lacked any clear nuclear localization potential. It is therefore likely that specific nuclear localization signals within conjugative proteins are not essential for nuclear transformation *per se*, although they may assist in *efficient* plasmid transmission.

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Chapter 1: A Recent History of Trans-kingdom Conjugation*

ABSTRACT

Conjugation is a mechanism of horizontal gene transfer (HGT) first observed between bacteria. The conjugative mechanism appears to be analogous, and sometimes homologous, to other means of transferring genes from bacteria to possibly members of every biological kingdom. As such, conjugative mechanisms of DNA transfer are necessary for a host of spectacular phenotypes such as symbiosis, virulence and antibiotic resistance. The conjugative mechanism is also related to the means of translocating and transferring proteins from bacteria to other species. Thus, this nearly generic form of macromolecular transport may move genes and other molecules across species boundaries. Some of these molecules may have immediate effects (e.g. through pathogenesis) and some lasting effects (e.g. through inheritance). There is even evidence that inheritable effects can be caused by transferred proteins. Interest in HGT, previously considered on the fringe, has increased dramatically due to the realization that HGT is not an anomaly but a biological fundamental.

* This chapter is a modified version of Ferguson *et al.* (2002) in 'Horizontal Gene Transfer' 2nd Edition, Eds. Syvanen, M. and Kado, C.I. p. 3-7.

The idea that genes are transferred at any appreciable frequency between species has evolved from one scorned by molecular phylogenists to a mainstream concept. Previously, only frustrated phylogenists would dwell on the odd DNA sequence that could unlace the bootstrap analysis (Gogarten *et al.*, 1999). Whole chromosome sequencing of organisms, however, is beginning to validate the concept that genomes are littered with “carcasses” of DNA from other species—some genes remaining functional and neutral, beneficial, or deleterious to the host, and some slowly fading away into the background average G+C content of the new host.

The extent of horizontal gene transfer (HGT) between organisms is difficult to determine for two main reasons. DNA sequence information is, firstly, limited by the simplicity of the four letter code and secondly, by the constraints on the sequence when it must reproduce in synchrony with the host (Heinemann and Roughan, 2000; Heinemann, 2000b). Thus, the mechanisms of HGT as well as bioinformatic tools are required to quantify the extent of HGT.

The renaissance in HGT thinking brought about by bioinformatics has a history and origin different to the mechanism studies. Mechanistic studies identify the means by which genes move between two neighbours that may or may not share a vertical lineage. Studies describing the gene transfer mediated by viruses, plasmids, transposons and transformation are much older than bioinformatics. Mechanism studies did not make HGT a mainstream concept, though, because they were considered “laboratory phenomenon” or “interesting exception to the rule for most genes or most organisms” by many. The mechanism studies did, however, open imaginations to the potential for HGT and legitimized those who subjected it to serious study.

This introductory chapter will focus on gene transfer between prokaryotes and eukaryotes by mechanisms that are identical, or similar, to bacterial conjugation. The review will not be a systematic account of all the literature relevant to HGT and conjugation. Instead, it will focus on publications that represent unambiguous

conflations of ideas that led to HGT becoming an independent phenomenon for study and established bacterial conjugation as a central, general, mechanism for interkingdom gene transfer (Amábile-Cuevas and Chicurel, 1992; Heinemann, 1992). An abbreviated history of the merger between HGT by conjugation and crown gall disease in plants is followed by a discussion of bacterial conjugation as a paradigm of interkingdom macromolecular exchange mechanistically connected to pathogenesis.

By the mid-20th Century, interspecies gene transfer was recognized as an important means by which bacteria acquired antibiotic resistance. Those findings, as indeed most early studies in gene transfer, remained focused on the particular genes or organisms of interest. From review of the literature it appears that a change in thinking about HGT was gaining momentum in the late 1960s. Subsequently, a number of studies examined HGT as a possible phenomenon in its own right, without need of allusion to important organismal adaptations, the success of pathogens (e.g. viruses and *Agrobacterium tumefaciens*), or the exception to the rule that all prokaryotic biology can appear to be to botanists and zoologists!

The convergence of interkingdom DNA transfer and crown gall.

A. tumefaciens was clearly linked to crown gall tumors in some plants long before the 1960s (references in Stroun *et al.*, 1970; Nester and Kosuge, 1981; Zhu *et al.*, 2000). However, the seminal clues that the nature of the disease was inseparable from DNA transfer to the host emerged in that decade. Work by Kerr demonstrated that *A. tumefaciens* virulence characters were transmitted between bacteria, by an unknown mechanism (Kerr, 1969). In the late 1970s, the DNA that caused gall formation, T-DNA, would be identified as a component of a conjugative plasmid, called Ti, in *A. tumefaciens* (Nester and Kosuge, 1981). The T-DNA was subsequently found integrated into plant chromosomes (Zambryski *et al.*, 1980; Thomashow *et al.*, 1980; Yadav *et al.*, 1980).

The search for T-DNA illustrates two different approaches to the study of interkingdom gene transfer operating simultaneously. One group of researchers, which we arbitrarily call the generalists, was dominated by the sense that HGT was a phenomenon independent of the particular biology of the donor and recipient organisms, such as the biology of the phytopathogen *A. tumefaciens* and its potential plant hosts. The other, which we refer to as the specialists, used the power of the causal relationship between *A. tumefaciens* and the gall tissue to discover HGT. The two approaches had complementary strengths and both endured the inevitable false positive and negative results that accumulate whenever techniques are pushed to their extreme limits of sensitivity.

The path to the discovery of the discrete DNA sequences transferred from *A. tumefaciens* to the host, and even to other soil bacteria, was itself a study in the limits of the contemporary molecular techniques. The pioneers at the roots of the crown gall mystery during the 60s and 70s were also at the leading edge of molecular biology and biochemistry. From such an edge, there is the risk of accumulating negative results, that is for example, of not seeing DNA transfer (see below). New techniques also require refinement to distinguish between the noise at their limits of detection and true signals. The results of these early studies were consistently “equivocal, but collectively they suggested that bacterial nucleic acids might play a role in tumorigenesis” (p. 186 Drlica and Kado, 1975).

Generalists and specialists.

Both generalists and specialists were reporting the transfer of bacterial nucleic acids and possibly proteins to eukaryotes by the late 1960s. The nucleic acids were invariably pursued in bacteria-free tissues by hybridization (references in Drlica and Kado, 1975) or hybridization and density centrifugation (e.g. Stroun and Anker, 1973; Stroun *et al.*, 1970; Stroun and Anker, 1971).

The conclusiveness of the hybridization method itself, however, was systematically challenged (Drlica and Kado, 1975). Hybridization methods used to demonstrate the presence of bacterial DNA in eukaryotes were often flawed because a control measurement of hybrid thermal stabilities or dissociation profiles was omitted (Kado and Lurquin, 1976; Drlica and Kado, 1974; Chilton *et al.*, 1974). With improved techniques applied later in the 1970s, *A. tumefaciens* nucleic acids were not detected in tumors (Drlica and Kado, 1974; Chilton *et al.*, 1974). The data of some groups were unable to be reproduced at this experimental standard (for an excellent discussion on the technology of the period, see Drlica and Kado, 1974).

Why did some detect nucleic acids while others did not? One possible explanation is that the sporadic claims of nucleic acid detection were artifacts generated by techniques pushed to their limits. A second possibility is that the practitioners of state-of-the-art techniques are important contributors to detection limits. A third possibility is experimental design. Of course, these three possibilities are not mutually exclusive and cannot be distinguished retrospectively.

With the increase in rigor applied to hybridization experiments came an increase in the precision for calculating the detection limits of the techniques (Drlica and Kado, 1974; 1975; Kado and Lurquin, 1976). Chilton *et al.'s* (Chilton *et al.*, 1974) DNA-DNA hybridization technique, for example, limited detection to one bacterial genome per three diploid plant genomes and "would not detect single or even multiple copies of a small specific fraction (<5%) of the bacterial...genome in tumor DNA" (p. 3675). Such famous negative results cannot, unfortunately, be directly compared to all reported positive detection of nucleic acids because of differences in determining the sensitivities of the techniques. Thus, history cannot distinguish between sporadic artifacts and individual experimenters as explanations for different results from all contemporary experiments.

Some groups monitored the production of bacteria-specific nucleic acids in eukaryotic tissues (e.g. Stroun *et al.*, 1970). Although these studies were also not

above the criticisms leveled against other hybridization studies and were not consistently reproduced (discussed in Drlica and Kado, 1975), ongoing RNA synthesis potentially provided access to larger quantities of nucleic acids complementary to the probe. In contrast, those groups searching only for transferred bacterial DNA were limited by the small number of copies of those sequences in preparations of eukaryotic genomes. History cannot distinguish between possible sporadic artifacts and differences in experimental design as the explanation for different data from all the different experimenters.

Some generalists introduced further confusion when they reported that DNA transfer occurred from not just *A. tumefaciens*, but also *Escherichia coli, Bacillus subtilis* and *Pseudomonas fluorescens* to both plants and animals. Hence, “The relationship of (these observations) to the crown gall disease (was) ambiguous” (p. 191 Drlica and Kado, 1975). Since only *A. tumefaciens* induced tumors, the mechanism of putative nucleic acid transfers from these other bacteria may have been irrelevant to that conducted by *A. tumefaciens* when it induced tumors.

The generalist view was to be eclipsed by the finding of particular T-DNA sequences in plants and the characterization of a mechanism that could account for its transfer. T-DNA transfer would, for a time, serve as the paradigm of interkingdom gene transfer systems. The generality of HGT would be revived in the 1980s by the finding that bacterial conjugative plasmids and T-DNA were different DNA transferred by the same mechanism (Sprague, 1991; Heinemann, 1991), providing retrospective credence to generalists’ claims if not vindication of early experiments.

Critical experimental limits to HGT detection.

Until recently, interkingdom DNA transfer has been mostly observed through the isolation of phenotypically recombinant organisms (i.e., gene transmission). DNA transfer can be inferred from any instance in which donor genes are recovered

from recipient organisms. This is usually accomplished by selecting recombinant phenotypes. Such phenotypes are the complex product of gene transfer and subsequent stabilization in the germ line of the recipient. Gene transfer is likely not the limiting event in most instances of gene transmission (Matic *et al.*, 1996; Heinemann, 1991). Since inheritable phenotypes or stably maintained DNA sequences remain the easiest way to detect transferred genes, the importance of gene transmission in biasing inferences of the rate and extent of HGT cannot be ignored. In fact, the general reliance on observing recombinant phenotypes or isolating transferred DNA from offspring underestimates HGT (Heinemann, 2000b; Heinemann and Roughan, 2000; Drlica and Kado, 1974; Chilton *et al.*, 1974).

Several authors over the years have emphasized the importance of distinguishing between gene transfer and transmission to avoid instilling a bias in experimental design and interpretation (reviewed in Heinemann, 1991). Clark and Warren made the most systematic justification for the terminology (Clark and Warren, 1979). The first authors to demonstrate the generality of interkingdom conjugation openly acknowledged the influence of that review on their experimental design (Figure 1) (Heinemann and Sprague Jr, 1989). Confusion between transfer and transmission may have similarly delayed discovery of transfer of DNA from *A. tumefaciens* to plants outside the bacterium's infectious host range (Grimsley *et al.*, 1987).

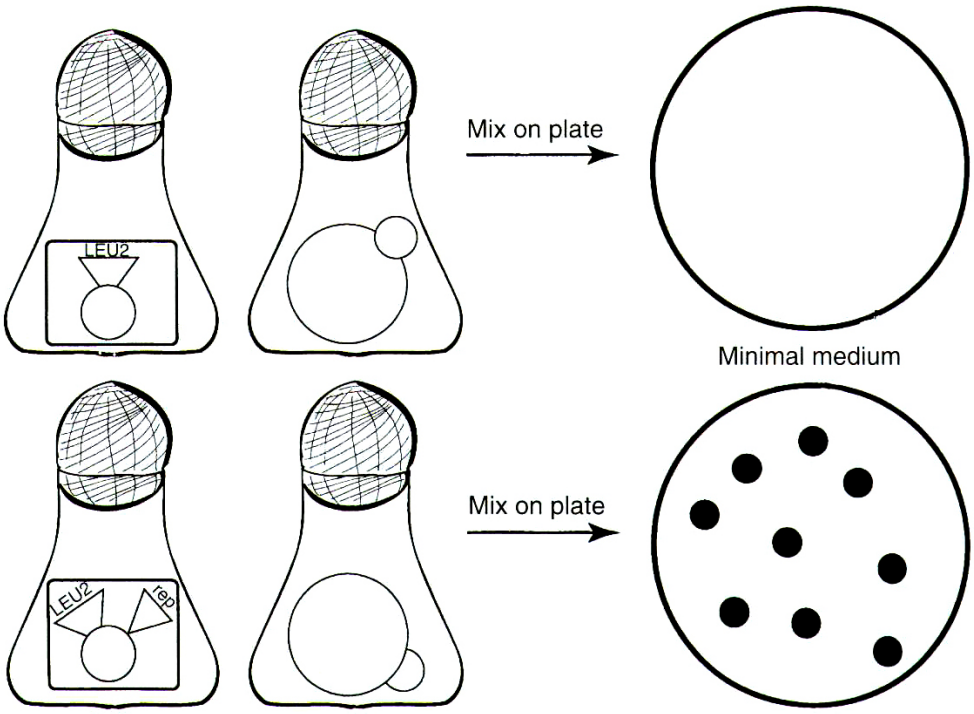

FIG.1. Illustration of the original experiment demonstrating DNA transfer from bacteria to yeast by conjugation. The rationale for the experiment was that DNA transfer was more generic than could be detected by DNA amplification or the formation of recombinant organisms, which requires DNA transmission (Heinemann and Sprague Jr, 1989). As a test, specially constructed donor bacteria (rectangles) were mixed with genotypically marked recipient yeast (circles with “buds”) and plated on medium (large open circles) permissive to the growth of only recombinant yeast. The conjugative plasmids (open circles inside bacteria) were modified to carry either the selectable yeast LEU2 gene or both LEU2 and a DNA sequence that permits replication of extrachromosomal DNA in yeast (rep). Colonies of yeast recombinants (solid black circles) were recovered at a frequency of up to 10% (per donor bacterium) when the plasmid carried yeast-specific replication sequences. Since the DNA introduced into the conjugative plasmids was not responsible for DNA transfer (Bates *et al.*, 1998; Heinemann, 1991; Heinemann and Sprague Jr, 1989), these experiments unequivocally demonstrated that transmission (necessary for detecting recombinants because the DNA is subsequently inherited vertically) was a poor indicator of transfer and the absence of experimentally demonstrated transmission did not imply the absence of DNA transfer.

To further illustrate the importance of distinguishing transfer from transmission, consider the recent report of a DNA virus, that infects animals, evolving from an RNA virus that infects plants (Gibbs and Weiller, 1999). The plant virus must have been able to transfer to animals (but caused no obvious phenotype). Once inside the animal, its genome may have been converted into DNA and probably acquired a portion of an animal DNA virus, conferring upon the recombinant the ability to be inherited in animals. The many transfer events preceding the evolution of the new variant virus were not detected by selecting or observing a recombinant animal, and likely would not have been detected even with current DNA amplification technologies. The transmission event could be detected, but provides no quantitative information about the frequency of transfers of the original DNA virus to plants.

Furthermore, transferred nucleic acids can be retained by recombination even if whole genes are not inherited (reviewed in Heinemann, 1991; Matic *et al.*, 1996). The extent of this recombination can be masked by the selectivity of homologous recombination enzymes that eliminate long tracts of dissimilar nucleotide sequences better than short tracts (Rayssiguier *et al.*, 1989; Heinemann and Roughan, 2000). Certain environments and mutations that reduce the activity of mismatch repair systems in particular have the effect of reducing selectivity (Matic *et al.*, 1995; Heinemann, 1999b; Vulic *et al.*, 1999). Recombination events resulting in the incorporation of short tracts of DNA, even over sequences of extreme genetic divergence, can be difficult or impossible to identify by analysis of DNA sequences (Heinemann and Roughan, 2000).

Conjugation as a paradigm system of interkingdom DNA transfer.

The first indication that bacterial conjugation described a general mechanism of interkingdom gene transfer came from the suggestion that certain DNA intermediates observed in *A. tumefaciens* resembled hypothetical DNA intermediates in bacterial conjugation (Stachel *et al.*, 1986). In hindsight, that

connection was probably better informed by inspiration than actual data, but nevertheless has withstood significant test.

Conjugation.

Bacterial conjugation in its broadest sense has been extensively reviewed, so only a brief description will be provided here (Frost, 2000; Heinemann, 1992; 1998). The focus in this review is on the paradigm conjugative systems defined by the IncP and IncF plasmid groups.

Conjugation mediated by these plasmids requires, at a minimum, a *cis*-acting DNA sequence called the origin of transfer (*oriT*). All other functions (called *tra*) act *in trans* thus allowing plasmids with all *trans*-acting functions to also transfer plasmids with no or a few *trans*-acting functions (Heinemann, 1992). The *trans*-acting gene products are divided further into those involved in DNA metabolism (and are usually specific to a particular *oriT*) and those involved in DNA transport and cell-cell interactions (and thus will interact with a greater range of other plasmids). The conjugative genes specific to DNA metabolism introduce a nick at *oriT* and initiate the unwinding and concomitant transfer of DNA to a recipient cell. Both strands are used as templates for the synthesis of a complementary strand, one in the donor cell and one in the recipient.

Single-stranded plasmid DNA (ssDNA) has been captured in recipient cells, confirming the mechanism of plasmid mobilization (Freifelder and Freifelder, 1968). The DNA is recircularized in the recipient. The transport apparatus has not been described biochemically (Heinemann, 2000a), but the genes necessary for forming the apparatus are all plasmid-encoded (Heinemann and Ankenbauer, 1993; Heinemann *et al.*, 1996).

T-DNA is interkingdom conjugation.

This uncontroversial model of the conjugative process grounded a model of T-DNA mobilization and transfer proposed by Stachel *et al.* (Stachel *et al.*, 1986). Their experiment involved isolating DNA of the T-DNA region from *A. tumefaciens* (not the plant) after it was induced to prepare the T-DNA for transfer. They provided convincing evidence that linear ssDNA strands defined by the left and right borders of the T-DNA region accumulated in induced bacteria, and that Ti plasmids from induced bacteria had nicks in the border sequences on the strand corresponding to the liberated T-DNA.

It appeared to Stachel *et al.* that the left and right borders of the T-DNA region, which are characterized as direct repeats, functioned like *oriT* sequences. Nicking and unwinding liberated only the DNA between the nicks, rather than a strand of DNA the length of the Ti plasmid. When the transfer process could not be completed, the T-DNA accumulated in the bacterium.

However, the phenomenology in this study differed from the molecular biology of conjugation in important ways. First, hypothetical ssDNA transfer intermediates *do not* accumulate in bacteria that hold conjugative plasmids even when constitutively induced (reviewed in Christie, 1997b). Second, the conjugative ssDNA was isolated from bacterial recipients (Freifelder and Freifelder, 1968); the so-called T-DNA in the Stachel *et al*. study was never recovered from plants (Stachel *et al.*, 1986). Third, there existed no evidence at the time that the DNA between tandemly repeated *oriT*s would be liberated during mobilization. Whereas it was shown subsequently that tandem *oriT* repeats do result in mobilization-specific DNA instability in some plasmids (Bhattacharjee *et al.*, 1992; Furuya and Komano, 2000), the repeat of IncP *oriT*s, which are thought to be the closest relatives of the T-DNA borders (Waters and Guiney, 1993; Waters *et al.*, 1991), does not result in mobilization-specific liberation of intervening DNA (Heinemann and Schreiber, pers. obs.).

Nevertheless, the model has been vindicated by several subsequent genetic tests (Christie, 2000; Lessl and Lanka, 1994). First, T-DNA recombination experiments within plant cells provide evidence that T-DNA is transferred, and enters the nucleus, single-stranded (Tinland *et al.*, 1994). Furthermore, single-stranded T-DNA intermediates have been recovered from plant protoplasts (Yusibov *et al.*, 1994). Second, the processing reaction between the *cis*-acting border repeat sequences and its putative nick-ase (*virD2*) could be replaced with the *oriT* and its cognate nick-ase (*mobA*) from the IncQ plasmid RSF1010 (Buchanan-Wollaston *et al.*, 1987). Third, RSF1010 transmission between Agrobacteria was found to be dependent on the other Ti-encoded genes *virA*, *virG, virB4, virB7* and *virD4* (Beijersbergen *et al.*, 1992). Thus, the *vir* genes, originally identified because they were necessary for virulence, can substitute for *tra* in mediation of conjugative plasmid transfer.

The ability to mix and match genetic requirements of bacterial conjugation and Ti-mediated virulence is consistent with the structural similarities of conjugative and virulence genes (Table 1). The *oriT* region of IncP plasmids is homologous to the T-DNA borders (Waters *et al.*, 1991; Frost, 2000), while the *oriT* of the Ti plasmid is homologous to the IncQ *oriT* (Farrand *et al.*, 1996). Many macromolecular transport systems appear to be composed of gene products homologous to the *tra* functions of conjugative plasmids, including the *vir* genes and type IV protein secretion systems in *Bordetella pertussis, Helicobacter pylori* and *Legionella pneumophila* (Christie and Vogel, 2000; Christie, 2001; Frost, 2000) (Tables 1 and 2).

TABLE 1. *A. tumefaciens* T-DNA transfer genes that are homologous to genes required for conjugation, protein transfer and virulence in a range of gram-negative bacteria

Proposed functions of *vir* genes required for T-DNA transfer from *A. tumefaciens* to plants[a]		*vir* homologues on conjugative plasmids					*vir* homologues involved in protein transfer/virulence				*vir* homologues with as yet unknown function	
		IncF[a]	IncP[a]	pTiC58 (*tra*)[b]	IncW[a]	IncN[a]	*B. pertussis*[a]	*B. suis*[c] *B. abortis*[g]	*L. pneumo-phila* (*icm/dot*)[d]	*H. pylori* (*cag*)[a]	*L. pneumo-phila* (*lvh*)[e]	*R. prowazekii*[e]
virB1	Trans-glycosylase	*orf169*	*trbN*			*traL*		*virB1*				
virB2	Pilin subunit	*traA*	*trbC*	*trbC*	*trwL*	*traM*	*ptlA*	*virB2*			*lvhB2*	
virB3		*traL*	*trbD*	*trbD*	*trwM*	*traA*	*ptlB*	*virB3*			*lvhB3*	
virB4	ATPase, transport activation	*traC*	*trbE*	*trbE*	*trwK*	*traB*	*ptlC*	*virB4*		*cagE*	*lvhB4*	*virB4*
virB5	Pilin subunit	*traE*	*trbF*	*trbF*	*trwJ*	*traC*		*virB5*			*lvhB5*	
virB6	Candidate pore former		*trbL*		*trwI*	*traD*	*ptlD*	*virB6*			*lvhB6*	
virB7	Transporter assembly				*trwH*	*traN*	*ptlI*	*virB7*				*rp288*
virB8					*trwG*	*traE*	*ptlE*	*virB8*			*lvhB8*	*rp289*
virB9	Transporter assembly				*trwF*	*traO*	*ptlF*	*virB9*		*orf15*	*lvhB9*	*rpB9*
virB10	Coupler of inner and outer membrane subcomplexes	*traB*	*trbI*	*trbI*	*trwE*	*traF*	*ptlG*	*virB10*	*dotG/icmE*	*orf13*	*lvhB10*	*rpB10*
virB11	ATPase, transport activator		*trbB*	*trbB*	*trwD*	*traG*	*ptlH*	*virB11*	*dotB*	*orf11*	*lvhB11*	*rpB11*
virD4	ATPase, coupler of DNA processing and transport systems	*traD*	*traG*		*trwB*					*orf10*	*lvhD4*	*rpD4*

virD2	Site-specific single-stranded nicking at the right and left borders		*traI**		
Right and left borders	Site of VirD2 nicking		*oriT* [f]		

Table adapted from (Christie, 1997b).

[a] (Christie, 1997b; Christie and Vogel, 2000) [b] (Li *et al.*, 1998) [c] (O'Callaghan *et al.*, 1999) [d] (Frost, 2000)[e] (Segal *et al.*, 1999) [f] (Waters *et al.*, 1991) [g] (Sieira *et al.*, 2000)

* Functional homology (Pansegrau *et al.*, 1993).

Conjugation is sufficient for interkingdom gene transmission.

A surprise to the crown gall groups was the finding that the transfer of DNA from *A. tumefaciens* to plants was related in part to bacterial conjugation. Meanwhile, yeast studies were soon to show that conjugation could account for interkingdom DNA transfer and that the ability to conjugate with eukaryotic cells is not an evolutionary quirk of *A. tumefaciens* (Figure 2).

In 1989, bacteria were crossed with the yeast *Saccharomyces cerevisiae* using the same plasmids that mediated conjugation between bacteria (Heinemann and Sprague Jr, 1989) (Figure 1). *E. coli* transferred a plasmid marked with the *S. cerevisiae* replication origin 2μ and LEU2 gene, to yeast. Recombinant (Leu^+) yeast were only formed when the bacteria contained a conjugative plasmid able to mobilize the marker plasmid *in trans*. Formation of Leu^+ yeast recombinants was dependent on donor-recipient contact, donor viability, functional *oriT* and *mob* genes, and was independent of exogenous DNAse, indicating that the mechanism of gene transfer was not transformation. *E. coli*-yeast conjugation was subsequently found to be dependent on the same *tra* genes as required for conjugation between *E. coli*, with no additional plasmid-encoded requirements (Heinemann, 1991; Bates *et al.*, 1998).

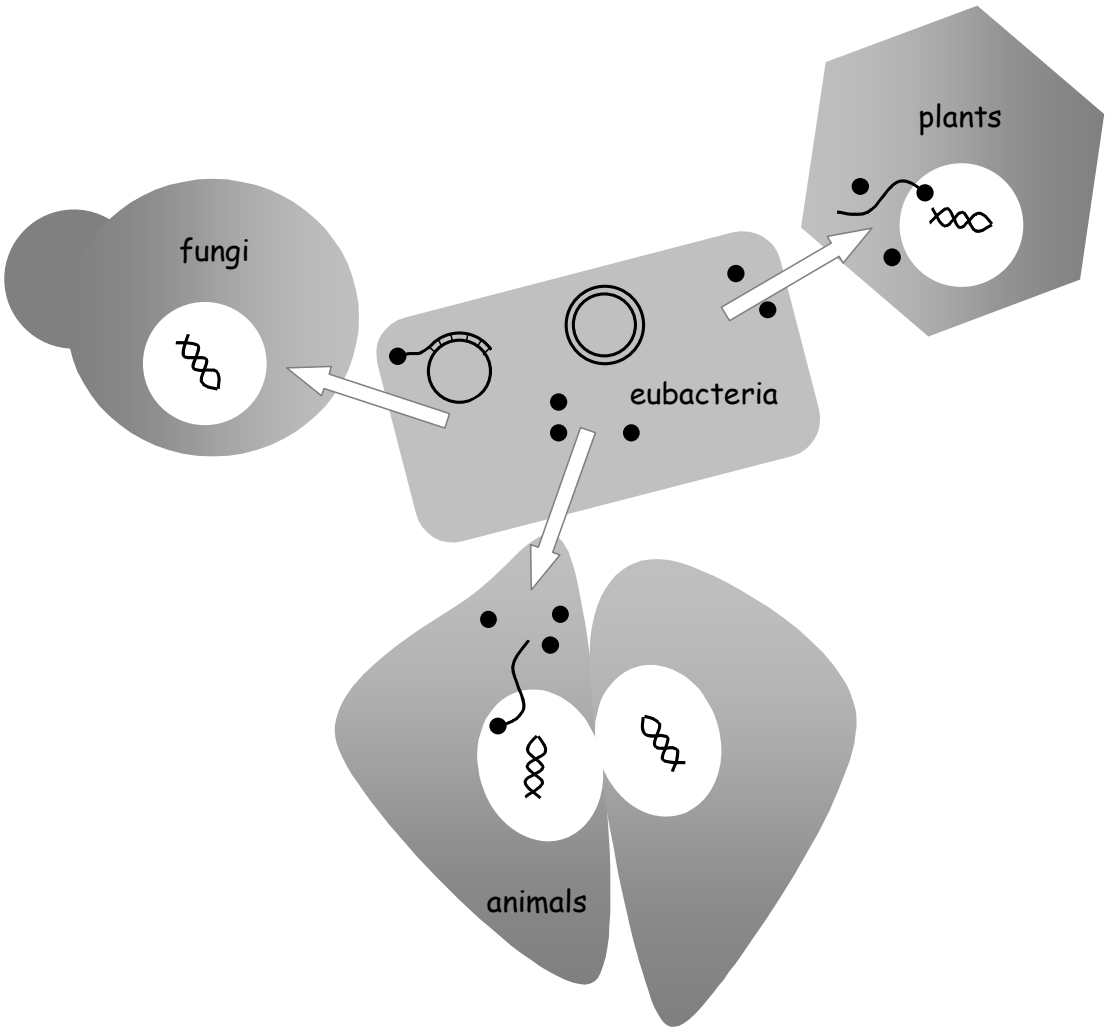

FIG. 2. Bacteria transfer DNA and proteins to plant, animal and fungal cells by similar and related mechanisms. Bacteria transfer DNA (solid lines and large open circles) to yeast, plant and animal cells by conjugation. Bacterial DNA is integrated into eukaryotic chromosomes (double helices) upon entering the nucleus (white ellipses). Proteins (solid black circles) are transferred to animal cells during pathogenesis. Conjugative plasmids have genes homologous to some genes required for virulence in many bacterial pathogens. Some of those homologous genes are known to be required for DNA or protein transfer.

These experiments suggested that DNA transfer from *E. coli* to *S. cerevisiae* occurred by a mechanism analogous to conjugation. The range of yeast able to serve as *E. coli* conjugal recipients has been extended to at least six evolutionary divergent genera (Heinemann, 1991; Hayman and Bolen, 1993; Inomata *et al.*, 1994). Unlike *A. tumefaciens* and plants, *E. coli* and yeast have no known ecological relationship and are not expected to have evolved such a specialized interaction. Therefore interkingdom gene transfer has few, if any, specific requirements evolved within the particular biology of the donor and recipient organism (although virulence and other phenotypes certainly do have specific requirements).

Interkingdom conjugation is not a species-specific phenomenon.

E. coli is not unique in its ability to conjugate with yeast. The T-DNA from *A. tumefaciens* also transferred to *S. cerevisiae,* but by *vir*-dependent conjugation (Bundock *et al.*, 1995). Using *URA3* as a selectable marker with or without the 2μ replication sequence between the T-DNA borders, the frequency of transmission of both replicative and integrative vectors was compared (Bundock *et al.*, 1995). Where transferred T-DNA could replicate autonomously, most transconjugants inherited the vector in its entirety. This was attributed to a failure of VirD2 to sometimes nick the left border, effectively creating a situation where the right border was the only *oriT*. Other transconjugants carried recirularized dsT-DNA molecules.

Interkingdom conjugation is not a plasmid-specific phenomenon.

Is the ability to conjugate with eukaryotic cells a particular feature of so-called ‘broad-host-range’ plasmids, such as the IncP family? Bates *et al.* (Bates *et al.*, 1998) compared the ability of conjugation functions from three incompatibility groups to transmit a marked shuttle vector to yeast. IncP plasmids transmitted the shuttle plasmid under conditions where transmission by the narrow-host-range

IncF and IncI1 plasmids was not detected (Bates *et al.*, 1998). In contrast, all plasmids were equally capable of transmitting the shuttle plasmid to *E. coli*.

Since recombinants were the only evidence of DNA transfer, it remains formally possible that some aspect of the IncP *tra* system enhances transmission by contributing to the ability of transferred DNA to be inherited. However, Heinemann and Sprague did observe F-mediated DNA transmission to yeast using an IncF plasmid derivative instead of mobilizing a shuttle plasmid *in trans* (Heinemann and Sprague Jr, 1989). The higher copy number of their F plasmid derivative may have contributed to the frequency of detectable DNA transmission (Bates *et al.*, 1998).

Conjugation mediates gene transfer between bacteria and mammalian cells

The range of conjugative recipients has recently been extended to include animal cells (Waters, 2001; Kunik *et al.*, 2001), making the known extent of the DNA exchange network inclusive of all kingdoms (Woese *et al.*, 1990) bar those of the Archaeal domain (Heinemann, 1991). Albeit at a low frequency, *A. tumefaciens* transferred T-DNA, encoding geneticin resistance, to human cells in culture (Kunik *et al.*, 2001). The requirements for *Agrobacterium*-human cell mating were shown to be the same as those for *Agrobacterium*-plant mating (i.e. a dependence on both attachment and *vir* genes), although interestingly, T-DNA transfer to animal cells was only partially dependent on acetosyringone (AS) induction. This first report of trans-kingdom conjugation involving animal cells was soon followed by a second. *E. coli* transferred an IncP plasmid to Chinese Hamster Ovary (CHO) cells by conjugation (Waters, 2001). A shuttle vector encoding the SV40 viral replication origin, various drug resistance markers, a viral antigen and the Green Fluorescent Protein (GFP) gene under control of a eukaryotic promoter, was mobilized to CHO cells *in trans* by an IncP helper plasmid. Transfer of the shuttle vector was dependent on *oriT*, the *mob* protein TraJ and the *tra* proteins TraF and G encoded on the helper plasmid. Transconjugant CHO cells were selected on the basis of viral antigen expression, drug resistance and green fluorescence and were recovered at a frequency of 10^{-4}-10^{-5} transconjugants per CHO recipient.

Conjugation as a convergence of macromolecular transport systems.

A. tumefaciens provided an anecdotal link between DNA transfer by conjugation and in pathogenesis. However, in that case, the disease was made possible by the genes transferred but DNA transfer was itself not causing the disease. It has become clear over the past decade that the DNA transport apparatus of conjugation is the ancestor, or at least a sibling (O'Callaghan *et al.*, 1999), of other macromolecular transport systems that are the raison d'etre of the disease. As mentioned above, type IV protein secretion genes are homologous to conjugation genes and the transport mechanism for both protein and DNA may be the same (Christie, 1997b; Winans *et al.*, 1996; Segal and Shuman, 1998a; Kirby and Isberg, 1998; Christie and Vogel, 2000; Christie, 2001).

Bioinformatics.

Many homologues of the Ti *virB* genes (B4, B9-11 and sometimes also *virD4*) are found on conjugative plasmids and on chromosomes, as inferred from similarities in sequence and organization. DNA transfer homologues include *tra* of IncN (Pohlman *et al.*, 1994) and Ti (Li *et al.*, 1998), *trb* of IncP and *trw* of IncW (Christie, 1997b; Kado, 1994) plasmids and the recent discovery of *avh* encoded on the *A. tumefaciens* C58 cryptic plasmid pAtC58 (Chen *et al.*, 2002). The *virB* genes have homologues in the pertussis toxin secretion system, *ptl* of *B. pertussis* (Weiss *et al.*, 1993; Covacci and Rappuoli, 1993; Farizo *et al.*, 1996; Shirasu and Kado, 1993). The *cag* pathogenicity island of *Helicobacter pylori,* implicated in contact-mediated secretion of proteins into epithelial cells, is homologous to *virB* (Covacci *et al.*, 1997; Christie, 1997a; Tummuru *et al.*, 1995; Censini *et al.*, 1996). Interestingly, *H. pylori* encodes a second type IV secretion system, not necessary for virulence but believed to be involved in IMPORT of extracellular DNA into the bacterium (Hofreuter and Haas, 2002; Smeets and Kusters, 2002). *virB* homologues have also been found in the chromosome of the obligate intracellular

parasite *Rickettsia prowazekii* (Andersson *et al.*, 1998), the arthropod intracellular pathogen *Wolbachia* sp. (Masui *et al.*, 2000), the human pathogen *Actinobacillus actinomycetemcomitans* (Kachlany *et al.*, 2000) and are essential for virulence in the intracellular pathogens *Brucella abortus* and *Brucella suis* (Sieira *et al.*, 2000; O'Callaghan *et al.*, 1999).

Relations between protein and DNA secretion systems is not restricted to *vir*. The *icm/dot* genes, essential for *L. pneumophila* survival and replication inside human alveolar macrophages, are homologous to conjugation genes from various plasmids (Vogel *et al.*, 1998; Purcell and Shuman, 1998; Segal *et al.*, 1998; Segal and Shuman, 1999; Segal and Shuman, 1997) (Table 2). Fourteen of the *icm/dot* genes are similar, both in sequence and in structural organization, to the *tra* region of IncI plasmid Col1b-P9 (Segal and Shuman, 1999), and *icmE* is homologous to *trbI* of IncP plasmid RK2.

Table 2. *tra* genes homologous to *icm/dot* genes [a].

L. pneumophila icm/dot	ColIb-P9 (IncI1)	RK2 (IncP)
icmT	*traK*	
icmS		
icmP	*trbA*	
icmO	*trbC*	
icmI	*traM*	
icmK	*traN*	
icmE		*trbI*
icmG	*traP*	
icmC	*traQ*	
icmD	*traR*	
icmJ	*traT*	
icmB	*traU*	
dotA	*traY*	
dotB	*traJ*	*trbB*
dotC	*traI*	
dotG	*traH*	

[a] Adapted from (Segal and Shuman, 1999).

Mechanism.

The link between protein and DNA secretory systems is also suggested by mechanistic studies. For example, radiolabeled DNA primases from both plasmids Col1b-P9 (IncI) and RP4 (IncP) (Rees and Wilkins, 1989; 1990; Wilkins and Thomas, 2000) and *E. coli*'s RecA protein (Heinemann, 1999a) were transferred to recipients during bacterial conjugation, possibly as protein-DNA complexes. Proteins were translocated to recipient cells by a conjugative mechanism, independently of DNA transfer however, in at least one of these cases (Wilkins and Thomas, 2000).

Likewise, the decreased stability of T-DNA transferred from *virE2* mutant bacterial donors is complemented by *in planta* expression of VirE2 protein (Citovsky *et al.*, 1992) and extracellularly by *virE2*$^{+}$ bacteria (Otten *et al.*, 1984), suggesting that VirE2 is also transferred into plants independently of T-DNA. In fact, VirE2, VirD2 and VirF may be secreted independently of both T-DNA and the *virB* genes, although tumorigenic *virB*-independent transfer of these proteins has not been demonstrated. The applicability of *virB*-independent secretion to the natural situation is additionally questionable since VirE2 and VirD2 were expressed at unnaturally high levels in these experiments (Chen *et al.*, 2000). It is possible that the VirB apparatus mediates secretion of proteins across the outer membrane only, with a second secretory pathway responsible for transport of VirD2 and VirE2 to the periplasm (Chen *et al.*, 2000) as is believed to be the case for secretion of the pertussis toxin (Farizo *et al.*, 2002).

Intriguingly, tumorigenicity is significantly inhibited when *A. tumefaciens* also carries the mobilizable plasmid RSF1010 (Binns *et al.*, 1995; Stahl *et al.*, 1998). Similarly, RSF1010 attenuates the virulence of *L. pneumophila* (Segal and Shuman, 1998b). In these two cases, the RSF1010:protein mobilization complex and the substrate of the virulence transport systems are thought to compete (Figure 3). That mutations in *mobA* suppress the effect of RSF1010 on *L.*

pneumophila virulence is consistent with this hypothesis (Segal and Shuman, 1998a). The *icm/dot* genes substitute for *tra* supplied *in trans* to transmit RSF1010 to recipient *L. pneumophila* by conjugation, indicating that the RSF1010:MobA complex is a substrate for the secretory system encoded by *icm/dot* (Vogel *et al.*, 1998; Segal and Shuman, 1998b; Segal *et al.*, 1998). The effect of RSF1010 on virulence could be failure to efficiently transport, as yet unidentified, effector proteins that alter vesicle targeting within the macrophage because they are displaced by the RSF1010:MobA complex (Segal and Shuman, 1998a). The *L. pneumophila virB* homologues *lvhB* do not complement the effect of *icmE/dotB* mutations on virulence, but do complement the effect of *icmE/dotB* mutations on conjugation (Segal *et al.*, 1999). Thus, the physical requirements for translocating the RSF1010:MobA complex and putative effector protein/s are not identical.

The effects of RSF1010 on *A. tumefaciens* tumorigenicity are suppressed by over-expression of *virB9*, *virB10* and *virB11* (Ward *et al.*, 1991), whose products are located in the cell membrane and form the putative conjugation pore (Christie, 1997b). Again, it has been suggested that an RSF1010:MobA complex may displace the T-DNA complex from the translocation apparatus due to the former's higher copy number, the constitutive presence of its processed form, greater affinity for the translocation complex or slow passage through the translocation pore (Binns *et al.*, 1995; Stahl *et al.*, 1998).

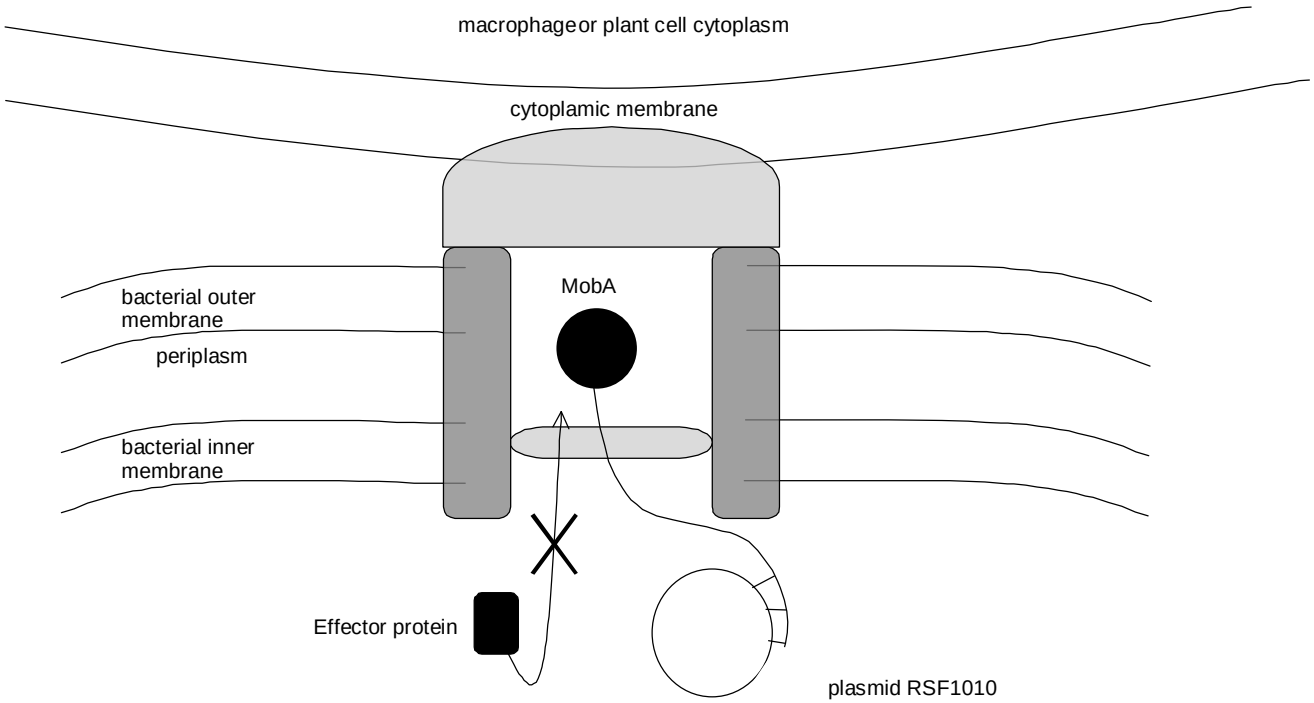

FIG. 3. The mobilizable IncQ plasmid RSF1010 inhibits transmission of T-DNA from *A. tumefaciens* to plant cells. (Adapted from Segal and Shuman, 1998a). Furthermore, RSF1010 inhibits the ability of *L. pneumophila* to evade fusion of its phagosome with lysosomes inside the macrophage. The *icm/dot* genes that are required to prevent lysozome fusion are also necessary for conjugative transfer of RSF1010. It has been proposed that *icm/dot* is a system that mediates secretion of proteins into the macrophage cytoplasm or phagosome during bacterial ingestion. The mobilized form of RSF1010 may inhibit virulence by competing with the natural substrate of these protein secretion systems.

The IncW plasmid pSa is an even stronger suppressor of tumorigenicity than RSF1010. Several lines of genetic evidence suggest that the *osa* gene product of pSa blocks protein VirE2 translocation (Chen and Kado, 1994; 1996; Lee *et al.*, 1999). *osa* was first identified as the gene sufficient to cause pSa abolition of oncogencity (Chen and Kado, 1994). The specific effect on VirE2 rather than a protein-DNA complex is supported by the observation that *osa* did not inhibit the conjugative transmission of the Ti plasmid.

The *osa* product also does not inhibit T-DNA transfer. *osa* did not suppress oncogenicity when expressed in *virE2* mutants as long as VirE2 was either supplied by separate donors through extracellular complementation or produced by the recipient plant cell (Lee *et al.*, 1999). The interesting ability for *virE2* mutants to be complemented extracellularly by separate VirE2 donors was suppressed, however, when *osa* was expressed in the protein donor (Lee *et al.*, 1999). Thus, the *osa* product specifically affects VirE2 translocation or function prior to T-DNA entry into the plant cell.

The effects of pSa and RSF1010 on oncogenicity are similar but not identical. Firstly, RSF1010 inhibits both VirE2 translocation and possibly T-DNA transfer, whereas pSa only prevents VirE2 translocation. Secondly, an RSF1010-protein complex is necessary for oncogenic suppression but only the *osa* gene product of pSa is required for suppression (Lee *et al.*, 1999). Thirdly, over-expression of VirB9, VirB10 and VirB11 suppresses the RSF1010 effect on tumorigenicity but not the *osa* effect. These apparent dissimilarities may reflect only quantitative differences in the RSF1010 and pSa mechanisms, since RSF1010 partially inhibits oncogenicity and pSa completely abolishes tumor formation (Lee *et al.*, 1999).

However, the RSF1010 and pSa effects may have different mechanistic explanations. As discussed above, VirE2, VirD2 and VirF proteins are transported across the inner membrane by a *virB24*- and *virD4*-independent mechanism (Chen *et al.*, 2000). The *osa* product, but not RSF1010, prevented VirE2, VirF and VirD2

from achieving normal periplasmic levels (Chen *et al.*, 2000). This suggests that the *osa* product and MobA-RSF1010 could inhibit VirE2 translocation at different steps. While MobA-RSF1010 may inhibit the directed translocation of proteins through the putative outer membrane pore, the *osa* product may inhibit translocation across the inner membrane. Such a model is consistent with both the inner membrane localization of Osa (Chen *et al.*, 1996) and the observation that VirB10, VirB11 and VirB12 over-expression did not restore tumor formation by *A. tumefaciens* carrying pSa (Lee *et al.*, 1999).

A new model for DNA transport.

The process of conjugative DNA transfer was recently likened to a coupling of two unrelated bacterial processes: a rolling-circle replication (RCR) system for DNA transfer and replication (Dtr) and a type IV secretion (T4S) system for translocation of a protein-DNA complex. While the link between Dtr and RCR systems and mating pore formation (Mpf) and T4S systems is clear, how the protein-DNA complex connects to the translocation complex remains to be determined. The role of adapting protein-DNA complexes to the T4SS has been assigned to the 'coupling protein', this being VirD4 in the prototypical T4SS of Ti. VirD4 homologues exist in all T4SS-related conjugation systems (e.g. TrwB of the IncW plasmid R388 and TraG of IncP plasmids) and are essential for DNA transfer. Coupling proteins bind DNA non-specifically and associate with the 'pilot protein' (VirD2 homologue), but there is no direct evidence, as yet, for an interaction between the coupling protein and components of the T4S system (Schröder *et al.*, 2002). Coupling proteins associate to form a hexameric particle with a central channel and these locate in the inner membrane, connecting the cytoplasm with the periplasm (Gomis-Rüth *et al.*, 2002; Llosa *et al.*, 2002). DNA may be transported through the translocation complex passively, by virtue of its covalent linkage to the pilot protein, the true substrate of the T4S apparatus. Since the coupling complex is essential for DNA transfer, it is likely to assist the transport of piloted DNA through the translocation complex; how this is achieved is not known.

VirD4 homologues are lacking from some T4S systems believed dedicated solely to the translocation of protein substrates (Christie and Vogel, 2000; Christie, 1997a), evoking the idea that coupling proteins are required specifically for adaptation of the T4S apparatus for the translocation of DNA. However, a number of T4S systems found in bacteria pathogens *do* encode VirD4 homologues and in *H. pylori*, at least, the VirD4 homologue HP0524 is essential for virulence (Schröder *et al.*, 2002). Thus, coupling proteins may fulfil a more general role in mediating substrate translocation through the bacterial inner membrane. Interestingly, the *plt* T4S system in *B. pertussis* lacks a VirD4 homologue and this may be correlated with the evolution of its two-step mechanism for toxin secretion, with translocation of pertussis toxin across the inner membrane being independent of T4S (Farizo *et al.*, 2002; Llosa *et al.*, 2002).

Furthering the link between protein and DNA translocation systems, *H. pylori* encodes two putative relaxases in addition to its DNA-binding VirD4 homologue (Llosa *et al.*, 2002). While these may represent the remnants of an ancestral DNA transfer system, from which the *cag* T4S system perhaps evolved, it is tempting to speculate that *H. pylori* may still possess the capacity for DNA transfer. In support of this, investigation of *H. pylori's* DNA transfer potential revealed a conjugation-like DNA transfer system, although, the genetic determinants have yet to be identified (Kuipers *et al.*, 1998).

What came first, protein or DNA transfer?

DNA and proteins are probably transferred between species by similar mechanisms. The effects of transferring non-nucleic acid molecules may sometimes be similar too; macromolecules, e.g. prions, other than nucleic acids possess gene-like qualities (Campbell, 1998; Heinemann and Roughan, 2000). Some proteins are not genes, but can influence epigenes that establish heritable phenotypes many generations after the protein has disappeared (Heinemann,

1999a). So conjugation may be a manifestation of protein secretion and, sometimes, protein secretion is another type of HGT.

Conclusion

HGT has established itself as a legitimate topic of study independently of the effects of the genes transferred on the biology of donor and recipient organisms. Nevertheless, the study of pathogens like *A. tumefaciens* and *L. pneumophila*, symbionts like *Rhizobium meliloti*, and phenotypes like antibiotic resistance and crown gall, have each contributed to the richness of the evidence supporting the notion that genes are less restricted by our notions of species sanctity than we have previously thought. In particular, the studies of bacterial conjugation, crown gall disease and protein secretion have provided extensive mechanistic insight into how DNA is exchanged between kingdoms, species and siblings.

Extensive similarities between genes identified as either virulence or conjugation determinants provided an early hint that macromolecular transport was a general phenomenon. Those early hints have been vindicated by demonstrations of genetic interchangeability between some determinants (complementation studies) and genetic conflict between others.

DNA is not special cargo but one of a number of molecules that might be transported by the same basic macromolecular transport systems. The ability to move molecules intercellularly has obvious implications for both single and multi-cellular organisms. Of immediate relevance are the diseases and recombinants that could arise from this nearly generic transport mechanism.

But what of the molecules being transferred? Plasmids and viruses, for example, make excellent evolutionary livings transferring between organisms, even evolving despite their effects on the host. Transfer alone might explain their existence (Cooper and Heinemann, 2000). Did these genetic entities evolve a means to

replicate by HGT, or was the existence of macromolecular transport enough for such semi-autonomous entities to evolve? Other kinds of molecules could transmit genetic information (Heinemann and Roughan, 2000). Could HGT be a mechanism for the evolution of genetic entities that are not nucleic acids?

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Chapter 2: Prologue

The former chapter focused on the transfer of DNA between unrelated cell types by conjugation. The topic of trans-kingdom conjugation will be revisited in Chapter 5: an investigation into the ability of putative conjugative escort proteins to mediate nuclear transformation. The ensuing bulk of this thesis is concerned with the transfer of plasmids between *bacteria* residing *within* cultured human cells (Chapters 3 and 4). Although at first glance these ideas may appear unlinked, they are unified under the theme of Horizontal Gene Transfer (HGT) and couple to the same evolutionary process - the acquisition of novelty - particularly, as the title of this thesis suggests, within the animal intracellular environment. This intermediary preamble, intended to connect the ideas of the introductory first chapter to those presented in Chapter 3, will explore some of these linking themes and explain the rationale behind this work.

The idea that bacteria can and have transferred DNA to animal cells over eons is supported phylogenetically (Smith *et al.*, 1992). Agreeably, these cases have a potential mechanistic basis: Doolittle (1998) proposed that Proto-eukaryotes, the predecessors of modern-day eukaryotic organisms, evolved a cytoskeleton for the purpose of ingesting solid food in the form of bacteria (Doolittle, 1998). These heretical ideas are not without precedent. Mitochondria likely arose from endosymbiotic proto-bacteria with consequent (well-documented) gene flow to the nucleus (Doolittle, 1998; de la Cruz and Davies, 2000; Margulis, 1970) and it is therefore highly likely that genes were transferred similarly to proto-eukaryotes from lysed intracellular bacteria ingested as food. Indeed, transformation of cultured mammalian cells with plasmid DNA released from bacteria residing within them occurs at a significant frequency (Courvalin *et al.*, 1995; Grillot-Courvalin *et al.*, 1998). Of interest to us was the possible potential of conjugative mechanisms to contribute to the flow of genes from intracellular bacteria to their host cells. This interest was inspired by the demonstration that bacteria could transfer DNA by

conjugation to other eukaryotic cells (Heinemann and Sprague Jr, 1989), and the discovery that bacterial protein secretion systems involved in pathogenesis are mechanistically analogous to (and genetically homologous to) conjugation systems (see chapter 1). In support of these concepts, gene transfer from bacteria to animal cells by conjugation was very recently demonstrated (Waters, 2001; Kunik *et al.*, 2001). For the sake of placing the ensuing experimental chapters within a literary context, the background to this project is elaborated within the following paragraphs.

What evidence is there that gene transfer from bacteria to eukaryotic organisms is of evolutionary importance? It was recently proposed that 0.5% of all human genes are derived from bacterial genes and that 223 of these were acquired by lateral transfer events occurring after the divergence of vertebrates from invertebrates (Lander *et al.*, 2001; and discussed by Ponting, 2001). The methodology upon which this assertion is based has been heavily criticized (Stanhope *et al.*, 2001; Salzberg *et al.*, 2001). Further to this, heritable gene transfer from bacteria to vertebrates would require genes to be transferred to germ line cells. Since no mechanism for germ line transformation with bacterial DNA is known, the possibility of historical gene transfer from bacteria to vertebrates is discounted by many (Stanhope *et al.*, 2001; Salzberg *et al.*, 2001).

Certainly, frequent bacteria-vertebrate gene transmission is difficult to reconcile with current theories on the evolution of life on earth. However, other seemingly incongruous happenings, which serve to distort the tree of life, enter the realms of possibility when considered in the light of new theories on the evolution of cells (Woese, 2002; Syvanen, 2002). Woese and Syvanen independently propose that the three modern cell types - Archea, Eukarya and Eubacteria – evolved not from a last common ancestor but as one of many independently-arising cell lineages (the majority of which became extinct) in a world where rampant HGT created a gene pool accessible by all. HGT is the source of novelty whereas vertical gene transfer and the process of slow change by mutation is the source of complexity and

specificity (Woese, 2000). The rate of evolution is predicted to slow as complexity increases since the degree of variation tolerable by an organism decreases. The evolution of complexity allowed each cell lineage to independently reach its "Darwinian threshold", the point at which a critical level of idiosyncrasy was reached, creating a barrier to HGT that was sufficient for speciation (Woese, 2002; Syvanen, 2002). That archea and eukarya are grouped together after the first branch from eubacteria therefore reflects not their vertical relatedness but that these groups reached their Darwinian threshold later than eubacteria. Prior to this point, it is likely that eukarya and archea continued to exchange genes freely with each other and with eubacteria, producing some gene lineages that are incongruous with the tenet that these two groups diverged from a common ancestor. That HGT was of fundamental importance in the evolution of modern-day cells is reflected in the universality of the genetic code; it is proposed that unification of the code was selected initially and maintained thereon in order to facilitate the exchange of genetic material (Syvanen, 2002; Woese, 2000).

Thus, gene transfer from bacteria to animal cells has been of significant evolutionary importance in the past and is likely still occurring in the present day (for examples see Sizemore *et al.*, 1997; 1995; Courvalin *et al.*, 1995; Grillot-Courvalin *et al.*, 1998; Dietrich *et al.*, 1998). But what of the reverse scenario? Evidence too exists for gene transfer from animal cells to both free-living bacteria (Smith *et al.*, 1992; Amábile-Cuevas and Chicurel, 1996) and obligate intracellular parasites (Wolf *et al.*, 1999). Notable examples include the *Escherichia coli* PapD, a chaperone protein involved in production of pili, believed to have derived from the lymphocyte differentiation antigen Leu-1/CD5, and the *Yersinia* YadA adhesion, a protein most similar to a group of eukaryotic signal transduction proteins (these cases are reviewed in Amábile-Cuevas and Chicurel, 1996). It is striking that both of these examples are genes involved in the close association of these bacteria with animal host cells; close contact would, of course, potentiate HGT.

Although the evolutionary impact of bacterial to animal cell gene transfer is independent of the transfer mechanism, we were interested specifically in the potential of conjugation to mediate gene transfer. As reviewed in detail in Chapter 1, conjugation may be the most universal of all horizontal gene transfer mechanisms and, in light of a recently discovered convergence of macromolecular transport systems, may have some relevance in the context of bacterial pathogen interactions with animal cells. Although speculative, the case of the *icm/dot* system in *Legionella pneumophila* (described in chapter 1) suggests a particularly compelling link between DNA transfer and pathogenicity since the *icm/dot* virulence genes complement the mobilizable IncQ plasmid RSF1010 for conjugative transfer to other bacteria (Segal *et al.*, 1998; Vogel *et al.*, 1998). By a mechanism believed similar to the inhibitory effect of RSF1010 on VirE2 secretion in *Agrobacterium tumefaciens* (Binns *et al.*, 1995), carriage of RSF1010 by wild-type *Legionella* compromises the bacterium's ability to evade fusion of its phagosome with lysosomes within the macrophage, a virulence strategy believed to be mediated by protein effectors translocated by the Icm/Dot secretion system (Segal and Shuman, 1998; Segal *et al.*, 1999). Our efforts (in collaboration with Dr. Howard Shuman, Columbia University) to demonstrate RSF1010 conjugative transfer from intracellular *L. pneumophila* to human and murine macrophages have so far been unsuccessful (data not shown here). The possibility that modern bacterial pathogens in their evolutionary past could, have and maybe still can transfer DNA to animal cells by type IV secretion-like mechanisms will be tested further.

To place the following experimental chapters within the above perspective, the original and underlying aim of the intracellular conjugation experiments (Chapter 3) was to demonstrate the functionality of the bacterial conjugative machinery in the animal intracellular environment. These experiments were designed within the context of a complementary research project within our laboratory, which aims to exploit the process of bacterial conjugation to direct plasmid DNA from intracellular or extracellular bacteria to the mitochondria of yeast and human cells.

The prospect of gene transfer between intracellular bacteria is, however, an interesting possibility in its own right. The interior of the animal cell represents a novel and potentially medically relevant environment for gene transfer. Horizontal (or lateral) gene transfer (HGT) is now indisputably the predominant driving force, if not the sole force, behind speciation and the evolution of novelty in bacteria (e.g. Lawrence, 1997; Ochman *et al.*, 2000; Lawrence and Ochman, 1998; Bäumler, 1997; de la Cruz and Davies, 2000; Lawrence and Roth, 1996; Reid *et al.*, 2000; Ochman, 2001; Woese, 2000). It is now conceded that even 'clonal' species such as the non naturally transformable *E. coli* evolve predominantly in 'leaps and bounds', by the acquisition of new traits from neighbouring species (compare Dykhuizen and Green, 1991; Smith, 1991 #282,with Guttman and Dykhuizen, 1994; Kudva *et al.*, 2002; Lawrence and Ochman, 1998; Bäumler, 1997; Reid *et al.*, 2000; Denamur *et al.*, 2000; Brown *et al.*, 2001; Vulic *et al.*, 1999). It is calculated that 15-18% of the *E. coli* genome has been acquired by HGT (in 234 independent events) since speciation from *Salmonella* species, that nearly 3000Kb of protein-coding sequence has been gained and lost from *E. coli* during this time (100 million years) and that none of the phenotypic characteristics that distinguish *E. coli* and *Salmonella* species can be attributed to point mutations and genetic drift (Lawrence, 1997; de la Cruz and Davies, 2000; Lawrence and Ochman, 1998). The extent of HGT has blurred the species barriers to the point where some pathogenic strains classed as *E. coli*, e.g. O157:H7, are more divergent in terms of the number of horizontally acquired 'adaptive units' and proportion of alien sequence from *E. coli* K12 than are the *Shigella* group who are granted their own genus (Ochman, 2001)! That the virulence determinants of distant pathogenic relatives show, in many cases, striking similarity is evidence for the supreme role of HGT in the dissemination of virulence throughout the bacterial kingdom (Cheetham and Katz, 1995).

To give a catalogue of all of the horizontally acquired adaptive traits in just *E. coli* and *Salmonella* sp. alone would be all-consuming and distracting from the flow of this discussion. However, to mention just one case in point, the mechanisms by

which *Salmonella typhimurium* and other distantly-related animal pathogens (e.g. *Yersinia* and *Shigella* species) invade animal cells are clear examples of how repeated horizontal acquisitions can sequentially build up a complex and finely-tuned repertoire of virulence strategies. In *Salmonella*, the type III secretion (TTS) genes necessary for invasion of epithelial cells are encoded on a large Pathogenicity Island called SPI-1 (for reviews see Ochman and Groisman, 1995; Groisman and Ochman, 1997; 1993; Bäumler *et al.*, 1998; Bäumler, 1997). SPI-1 was likely acquired prior to speciation of the *Salmonella* genus (but subsequent to divergence of *Salmonella* from *E. coli* and *Shigella* spp.). The invasive species of *Yersinia* and *Shigella* encode homologous TTS systems on virulence plasmids but neither of these is likely to have been the source of the *Salmonella* genes (Groisman and Ochman, 1993). Thus, it appears that all three genera acquired the TSS genes independently and from an unknown source.

Divergence of *Salmonella enterica* from *Salmonella bongori* was punctuated by the acquisition of a second pathogenicity island, SPI-2, recently found to be composed of two independently-acquired elements (Hensel *et al.*, 1999). SPI-2 encodes a second TTS system, necessary for systemic infection of mice and present in all of the subspecies of *S. enterica* (Bäumler *et al.*, 1998; Bäumler, 1997). Subsequent to the acquisition of SPI-2, *S. enterica* subspecies I, which includes the serovars Typhi and Typhimurium, acquired a number of additional virulence determinants which adapted this group to colonization of warm-blooded animal hosts. These include the *pef* fimbrial genes and *spv* genes necessary for intracellular replication during growth in the liver and spleen. Both *pef* and *spv* are encoded on a large F-like plasmid in some serovars of *S. enterica* subspecies I whereas in others *spv* is chromosomally located (Ahmer *et al.*, 1999; Boyd and Hartl, 1998). In the serovar Typhi, *spv* has been lost by deletion (Bäumler *et al.*, 1998). More recently, the evolution of virulence within pathogens like *S. typhimurium* has been associated with the acquisition of modular effector proteins; these being the translocated substrates of the TTS systems encoded on SPI-1 and SPI-2 (Mirold *et al.*, 1999; Hansen-Wester *et al.*, 2002; Miao and Miller, 2000; Mirold *et al.*, 2001). It is

supposed that the ability to 'mix and match' effectors with translocation systems allows bacteria to fine-tune their virulence strategies by sampling the genetic repertoire pre-adapted within other species (Miao and Miller, 1999; Mirold *et al.*, 1999). Newly acquired genes and gene clusters typically come under the 'surrogacy' of existing global regulatory systems in *Salmonella* to allow their co-ordinated regulation with other virulence genes in response to the appropriate environmental cues (Guiney *et al.*, 1995; Ochman *et al.*, 2000; Bäumler, 1997; Worley *et al.*, 2000). *Salmonella* global virulence regulators include the stationary phase sigma factor RpoS and the two-component regulatory systems PhoPQ and SsrAB. SsrAB regulates a large number of unrelated, horizontally acquired genes in *S. typhimurium,* including the SPI-2 TTS system (Worley *et al.*, 2000).

The recently acquired *Salmonella* TTS effector proteins allow us a glimpse of "evolution in action" since these are predominantly encoded on still-mobile lysogenic bacteriophage (Mirold *et al.*, 1999; Hansen-Wester *et al.*, 2002; Miao and Miller, 1999). Many other bacterial virulence determinants too are encoded on active phage and these instances are so numerous that the notion of phage playing only a minor role in evolution by HGT is seriously challenged (Ho *et al.*, 2002; Schicklmaier and Schmieger, 1995; Strauch *et al.*, 2001; Cheetham and Katz, 1995; Miao and Miller, 1999). Indeed, the larger SPIs themselves, of which there are at least 5, most likely evolved from phage (Hansen-Wester and Hensel, 2002). This is evident from the high incidence of juxtaposition of these structures (4 of the 5 major SPIs plus an additional 4 newly discovered elements) with tRNA genes, the preferred chromosomal integration sites for many lysogenic bacteriophage (Hansen-Wester and Hensel, 2002; Ochman *et al.*, 2000). Interestingly, 65 of 85 tested *S. typhimurium* strains were found to contain at least one lysogenic phage with many strains harbouring as many as four (Schicklmaier and Schmieger, 1995). 93.5% of the active phage were found to be generalized transducers, further supporting the potential for phage-mediated gene transfer in *Salmonella* evolution (Schicklmaier and Schmieger, 1995).

In contrast to the apparently ordered evolution of *Salmonella* species, the evolution of pathogenicity in *E. coli* appears to have arisen independently in each (or most) of the pathogenic *E. coli* lineages (STEC, EHEC, ETEC, UPEC, EIEC and *Shigella* sp.) and involves a range of plasmids, pathogenicity islands and lysogenic lambdoid phages such as those that encode the Shiga toxins Stx-1 and Stx-2 (Bäumler, 1997; Reid *et al.*, 2000; Ochman, 2001; Boerlin, 1999). Since non-pathogenic *E. coli* already contain many of the genes required for interaction with animal cells and survival in harsh environments, they can be described as 'pathogens in waiting' (Groisman and Ochman, 1997). Common *E. coli* lab strains became invasive when they received a large virulence plasmid from *S. flexneri*, suggesting that the transformation of a normal flora bacterium into a pathogen can be a one-step process (Sansonetti *et al.*, 1983). This indeed appears to have been the case (Bäumler, 1997; Reid *et al.*, 2000; Ochman, 2001; Boerlin, 1999).

That virulence evolves within, and spreads upon, Horizontally Mobile Elements (HMEs) is apparent, but the fundamental question is: why and how virulence and resistance genes accumulate on HMEs? Various hypotheses account for the clustering of the component genes for weakly selectable phenotypes into operons and the selective benefit for such traits to accumulate on HMEs (Lawrence, 1997; Jain *et al.*, 1999; Lawrence and Roth, 1996). Clustering prevents functional loss of modular traits by segregation. Horizontal mobility allows colonization of new species and reintroduction into those where the trait is lost. In contrast, strongly selected or highly complex traits (those demonstrating host-adapted specificity) locate on chromosomes since their uncoupling from organismal reproduction would likely be lethal (Levin and Bergstrom, 2000).

While most models of virulence and novel traits evolution are 'cell-centric' in that they study the effect of genes and traits on the cellular host, an alternative angle is presented by the 'competition model' (Cooper and Heinemann, 2000a). Here it is argued that natural selection acts at the level of the fastest reproducer. Although the products of natural selection are most readily observed as cellular phenotypes,

they were not necessarily selected at the cellular level. If an HME reproduces at a higher rate horizontally than it does vertically, then the reproduction of the HME is uncoupled from that of its host, allowing traits not necessarily beneficial to the host to arise (Heinemann and Roughan, 2000). Thus, the genes that evolve on HMEs do so for the benefit of the HME itself, not the host cell *per se* (Cooper and Heinemann, 2000a).

The most important mechanism of HGT, and the most central to this thesis, is conjugation. As discussed previously, conjugation may be the most universal of HGT mechanisms, mediating gene transfer both between dissimilar bacterial species and from bacteria to yeast, plant and animal cells. Indeed, conjugative plasmids are found abundantly in nature (Lundquist and Levin, 1986). The extent of conjugation in the environment, and thus its contribution to evolution, may have been underestimated and undervalued by laboratory studies undertaken with clonal liquid cultures. It has long been known that wild bacteria exist predominantly as complex multi-species communities growing as biofilms upon structured surfaces (Watnick and Kolter, 2000). Although a number of studies demonstrate bacterial conjugation within biofilms, (Dahlberg *et al.*, 1998; Christensen *et al.*, 1998) a recent study shows conjugative plasmids and biofilm-forming ability to be inextricably linked (Ghigo, 2001). Biofilm formation was mediated by the adherent properties of conjugative pili. Although conjugation was not necessary for biofilm formation *per se*, in the situation where small numbers of plasmid-carrying (P+) bacteria were challenged to initiate biofilm development from micro-colonies of biofilm-deficient (P-) bacteria, conjugation was indeed found to be a prerequisite. This was attributed to the phenomenon of "epidemic spread" (Lundquist and Levin, 1986). The close association of bacteria in a biofilm facilitated conjugation (Ghigo, 2001). While pili synthesis is normally repressed in a population of P+ bacteria, in nascent transconjugants pili synthesis is transiently derepressed, encouraging plasmids to “sweep” through susceptible populations (Lundquist and Levin, 1986). In fact, many of the natural conjugative plasmids tested in the study were initially found to be deficient in biofilm initiation when carried within a clonal P+ population

(Ghigo, 2001). However, in mixed populations, epidemic spread was sufficient to initiate formation of a biofilm. Thus, in natural situations, conjugative plasmids and conjugation itself may play an important role in determining the structure of microbial communities. Further, and pertinent to the previous discussion, plasmids encode mechanisms for increasing their own cell-to-cell spread and thus, contrary to former thinking, (Lundquist and Levin, 1986) can potentially be maintained in a population of bacteria by infectious transfer alone, a prerequisite for the evolution of plasmid-borne traits by HME-HME competition (Cooper and Heinemann, 2000a; Heinemann and Roughan, 2000).

The idea that plasmids themselves potentiate gene flux by encoding cell-cell contact mechanisms predates the discovery of the biofilm connection (Amábile-Cuevas and Chicurel, 1992). Examples of such cell-cell proximity mediated by plasmid genes include symbiosis, endosymbiosis and adherence. Interestingly, a number of bacterial pathogens use Type IV pili, homologous to, and likely evolved from, some conjugative pili (Yoshida *et al.*, 1999), for adherence to animal cells during pathogenesis (Zhang *et al.*, 2000; Kuehn, 1997; Yoshida *et al.*, 1999). Further to adherence, pili can mediate the communication between eukaryotic and bacterial cells that results in induction of virulence expression in the bacterium (Kuehn, 1997). It would be tempting to speculate that Type IV pili could also be involved in mediating conjugative plasmid transfer to animal cells.

Plasmids too are responsible for the evolution and rapid spread of antibiotic resistance (Amábile-Cuevas, 1993; Amábile-Cuevas and Cárdenas-García, 1996; Heinemann, 1999; Tenover and McGowan, 1996; Amábile-Cuevas and Chicurel, 1992). Like virulence genes, antibiotic resistance genes tend to accumulate on HMEs such as plasmids and transposons. The clustering of multiple antibiotic resistance genes alongside virulence genes and heavy metal and xenobiotic resistance genes on HMEs (e.g. Guerra *et al.*, 2002) is largely mediated by integrons, mobile elements optimized for the capture and subsequent movement of genes onto larger mobile elements (Amábile-Cuevas and Chicurel, 1992). It is the

tendency for resistance and virulence traits to cluster that makes antibiotic resistance so problematic. It had long been believed that reduction of antibiotic usage below a certain threshold would result in the eventual loss of antibiotic genes from a population of bacteria (Heinemann *et al.*, 2000; Salyers and Amábile-Cuevas, 1997). This belief is based on the assumption that carriage of antibiotic resistance genes comes at a price: a fitness cost that would make maintenance of antibiotic resistance genes unfavourable in the absence of a selective pressure for their maintenance. Within this assumption is nested a second: that selection for antibiotic resistance is mediated simply by the relevant antibiotic alone. Unfortunately, both of these assumptions have proven too simplistic.

Linkage of antibiotic resistance genes with other selectable traits causes resistance-bearing HMEs to be maintained in numerous antibiotic-free environments (Summers *et al.*, 1993; Wireman *et al.*, 1997; Threlfall *et al.*, 1994; Gyles *et al.*, 1977). In some cases, antibiotic resistance genes unexpectedly confer biochemical cross-resistance to other unrelated drugs and environmental chemicals (for a review see Heinemann *et al.*, 2000). Antibiotic resistance can be highly stable when linked with systems for plasmid maintenance within a population of bacteria. Moreover, populations losing a resistance or virulence plasmid can be rapidly recolonized by horizontal transfer from a reservoir population. On occasions when antibiotic resistance *is* associated with a fitness cost, secondary-site compensatory mutations can restore fitness without loss of antibiotic resistance (e.g. Giraud *et al.*, 2002; Björkman *et al.*, 1998; Lenski *et al.*, 1994). Indeed, secondary site mutations have been shown to be the predominant way in which bacteria overcome costs associated with resistance mutations (Björkman *et al.*, 1998; Schrag and Perrot, 1996). Finally, the evolutionary pressures that drive accumulation of resistance and virulence traits on HMEs and consequently support their maintenance may not be predictable from studying the effects of such traits at the cellular level. This is evident from the finding that antibiotic-mediated "death" of bacterial host cells does not necessarily prevent the horizontal dissemination of HMEs (Cooper and Heinemann, 2000b; Heinemann,

1999). If infectious transfer alone is sufficient to maintain an HME within a population of bacteria (Ghigo, 2001; Levin and Bergstrom, 2000) and competition between HMEs the main driving force behind their evolution (Cooper and Heinemann, 2000a), then the effects of HME-encoded traits on bacterial populations are merely secondary and thus difficult to predict.

It is, therefore, important to study HGT in natural environments, particularly those in which antibiotic resistance and virulence may evolve and disseminate. One such environment is the animal gut, and more specifically, the cells of the animal gut. Some studies have shown, or inferred, *in vivo* antibiotic resistance plasmid transfer between gut bacteria (e.g. Balis *et al.*, 1996; Anderson, 1975; Summers *et al.*, 1993). To our knowledge, our experiments (Chapters 3 and 4) are the first to show gene transfer between bacteria residing within animal cells. This finding allows us to present a new model (discussed in Chapter 3 and expanded in Chapter 4) for the simultaneous evolution of antibiotic resistance and virulence in bacteria.

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Chapter 3: Gene Transfer between *Salmonella enterica* Serovar Typhimurium inside Epithelial Cells*

ABSTRACT

Virulence and antibiotic resistance genes transfer between bacteria by bacterial conjugation. Conjugation also mediates gene transfer from bacteria to eukaryotic organisms including yeast and human cells. Predicting when and where genes transfer by conjugation can enhance understanding of the risks in the release of genetically modified organisms including those being developed for use as vaccines. We report here that *Salmonella enterica* Serovar Typhimurium conjugated inside cultured human cells. DNA transfer from donor to recipient bacteria was proportional to the probability that both types of bacteria occupied the same cell, dependent on viable and invasive bacteria, and dependent on plasmid *tra* genes. From the high frequencies of gene transfer between bacteria inside human cells we suggest that such gene transfers occur *in situ*. Implications of gene transfer between bacteria inside human cells, particularly in the context of antibiotic resistance, are discussed.

* This chapter is a modified version of Ferguson, G.C., Heinemann, J.A., and Kennedy, M.A. (2002) Gene transfer between *Salmonella enterica* Serovar Typhimurium inside epithelial cells. *J. Bacteriol.* **184,** 2235-2242.

INTRODUCTION

Genomic databases have confirmed the enormous contribution horizontal gene transfer (HGT) has made to the content and even the organization of bacterial genomes (for reviews see Gogarten and Olendzenski, 1999; Jain *et al.*, 1999; Lawrence and Ochman, 1998; Ochman *et al.*, 2000). One theory even attributes HGT with creation of the most famous of bacterial chromosomal structures, the operon (Lawrence, 1997). Genes transfer between bacteria via many different kinds of vectors, ranging from conjugative plasmids and conjugative transposons through integrons and *cis*-acting signal sequences, that mediate transformation of organisms such as *Haemophilus* and *Neisseria*, to viruses. These vectors are collectively referred to as horizontally mobile elements (HMEs) (Liebert *et al.*, 1999; Hall and Collis, 1995; Salyers *et al.*, 1995; Cheetham and Katz, 1995). If HGT is powerful enough to effect the organization of chromosomes, then what effect has it on the organization and function of the elements that mediate gene transfer?

A central question in bacterial evolution, and one of immediate medical interest, is why antibiotic resistance and new virulence determining genes are so often found on HMEs, rather than spreading on chromosomes of dominant clones of bacteria (Cooper and Heinemann, 2000a; Heinemann, 1999; Heinemann *et al.*, 2000; Tenover and McGowan, 1996). Further study of HGT in both the environment and in the laboratory is necessary to answer the questions about how chromosomes and HMEs attract different kinds of genes (Eberhard, 1989; 1990; Levin and Bergstrom, 2000).

We sought to address such questions of antibiotic resistance evolution through HGT between invasive bacteria that cause disease in humans. Many bacteria invade animal cells during pathogenesis (Finlay and Cossart, 1997). This ability to invade animal cells helps bacteria evade both the host immune system and antibiotics (Rakita, 1998). Eukaryotic cells protect internalized bacteria from other well-known stresses in a variety of ways. For example, some bacteria consumed

by protozoa are protected from environmental and chemical stresses (Barker and Brown, 1994) and many antibiotics penetrate animal cells poorly or accumulate insufficiently in the intracellular compartments to kill invasive bacteria (Rakita, 1998; Tulkens, 1991). Thus, we asked whether the cytoplasm of animal cells could be a niche for gene transfer between bacteria protected from antibiotics that might otherwise inhibit gene transmission.

We postulated that if bacteria conjugated in the intracellular environment, that could partially explain both the evolution of virulence and the spread of antibiotic resistance in invasive pathogens. Antibiotic-susceptible bacteria that have escaped the effects of antibiotics by entering human cells could acquire resistance genes from pathogens resident in the same cell, benign bacteria taken up by cells (Francis *et al.*, 1993), or through mixing with the normal flora upon re-emergence from the cell. Conversely, normally benign bacteria could acquire virulence genes intracellularly from resident pathogens, since many new virulence traits are plasmid-borne (Sansonetti *et al.*, 1982; Bacon *et al.*, 2000).

These ideas are made plausible by the observation of elevated frequencies of gene transfer between bacteria that are in association with protozoan cells (Schlimme *et al.*, 1997). Transmission of the conjugative plasmid RP4 between *Escherichia coli* strains increased three orders of magnitude in the presence of the protozoan *Tetrahymena pyriformis*, suggesting that plasmid transfer possibly occurred within digestive vacuoles.

Using antibiotic resistance genes as markers, and the invasive pathogen *Salmonella enterica* Serovar Typhimurium (hereon referred to as *S. typhimurium*) as a model, we sought to determine whether conjugative plasmids could be transmitted between bacteria inside cultured human cells.

How *S. typhimurium* invades epithelial cells has been well described (Ochman and Groisman, 1995; Finlay and Cossart, 1997; Hueck, 1998) and so is only

summarized briefly here. Epithelial cells take-up *Salmonella* when the bacteria translocate signal transduction-altering proteins to the cell by a Type III Secretion mechanism. Cytoskeletal rearrangements, membrane ruffling and bacterial uptake by macropinocytosis follow the translocation of bacterial proteins. Engulfed bacteria reside within membrane-bound vacuoles within the cell cytoplasm. Whereas some bacteria lyse the vacuole and are released into the cytoplasm, *S. typhimurium* remain vacuolar.

Bacteria within cultured mammalian cells are often distinguished from extracellular bacteria because intracellular bacteria are protected from gentamicin (Gm) in the medium (Isberg and Falkow, 1985; Lutwyche *et al.*, 1998). We exploited the property of human cells to exclude Gm to ensure that viable recipient and donor bacteria, introduced to a monolayer of cells at different times, could not form transconjugants in the extracellular medium. To our knowledge, this is the first demonstration of intracellular bacterial conjugation and demonstrates both the functionality of bacterial conjugative machinery in the intracellular environment and the potential for transfer of genes between intracellular bacteria, particularly in the presence of antibiotics.

MATERIALS AND METHODS

Bacteria. Δ*invA* strains were created by P22 transduction (Davis *et al.*, 1980) of the *invA61*::TnPhoA mutation from SB111 (Galán and Curtiss III, 1989)to wild-type *S. typhimurium* strains SL1344^{R} (Hoiseth and Stocker, 1981) and 14028^{R}-P (Bäumler *et al.*, 1996). Strains and plasmids are described in Table 1.

Cell culture. INT-407 cells (ATCC CCL 6) were cultured in Minimal Essential Medium with Earle's salts (MEM, Gibco) supplemented with 2mM Non-Essential Amino Acids (NEAA, Gibco), 2mM L-glutamine (Gibco) and 10% FBS (Fetal Bovine Serum, Gibco) and maintained at 37°C in a 10% CO_2 atmosphere.

Conjugation Experiments. For intracellular conjugation, 24-well (1.9cm^2) trays (Nunc) were inoculated with 4×10^5 tissue culture cells/ml, 0.5 ml per well and cells were grown to confluence (24-48 hours). In some experiments, cells were grown on 10mm membrane filter inserts with 2μm pore membranes (anopore™, Nunc). Prior to infection, monolayers were washed once with PBS (g/L: NaCl, 8; KCl, 0.2; Na_2HPO_4, 1.44; KH_2PO_4, 1.43; pH 7.4) and maintained in MEM supplemented with L-glutamine, NEAA and 1% FBS throughout the experiment.

Exponential phase cultures of recipient (plasmid-free) bacteria were washed and diluted in PBS to 1×10^8 bacteria/ml. Wells were inoculated with ~10^7 bacteria to give a multiplicity of infection (MOI) of ± 10, and spun at 317×g for 5 minutes. Following 2 hours incubation at 37°C (10% CO_2), monolayers were washed three times in PBS, then incubated in media with 100μg/ml Gm for one hour. The above procedure was repeated, substituting donor (plasmid-bearing) bacteria. One hour following the second Gm incubation period, the Gm concentration was lowered to 20μg/ml. After a further 3 hours the monolayers were washed with PBS and lysed with 0.5% deoxycholate to release intracellular bacteria (Konkel *et al.*, 1999).

TABLE 1. *S. typhimurium* strains and plasmids

Strain or plasmid	Relevant genotype and/or phenotype[a]	Source	Reference
Strains			
SL1344	Wild-type, *rpsL hisG46*	B.B. Finlay	(Hoiseth and Stocker, 1981)
SL1344^{N}	Spontaneous Nx^{r} mutant of SL1344	This study	
SL1344^{R}	Spontaneous Rf^{r} mutant of SL1344	This study	
14028-P	pSLT-cured derivative of 14028 wild-type	A.J. Bäumler	(Bäumler *et al.*, 1996)
BA770	Spontaneous Nx^{r} mutant of 14028-P	B.M.M. Ahmer	(Ahmer *et al.*, 1999)
14028^{R}-P	Spontaneous Rf^{r} mutant of 14028-P	This study	
SB111	SR11 *invA61*::TnPhoA	B.B. Finlay	(Galán and Curtiss III, 1989)
Δ*invA* SL1344^{R}	SL1344^{R} *invA61*::TnPhoA	This study	
Δ*invA* 14028^{R}-P	14028^{R}-P *invA61*::TnPhoA	This study	
Plasmids			
RP4	IncPα. (Kn^{r} Ap^{r} Tc^{r})		(Datta *et al.*, 1971)
F42::miniTn*10Kn*	F'*lac* carrying a Kn resistance gene on Tn*10*	I.J.Molineux	(Schmitt *et al.*, 1995)
Jp143	F'*lac traB*^{am} Kn^{r}		(Heinemann *et al.*, 1996)

[a]Nx^{r}, naladixic acid resistance; Rf^{r}, rifampicin resistance; Kn^{r}, kanamycin resistance; Ap^{r}, ampicillin resistance; Tc^{r}, tetracycline resistance; Tp^{r}, trimethoprim resistance.

Recombinant selection and transmission frequency calculations. Intracellular bacteria were enumerated by serial dilution in PBS and plating on LB (Gibco) agar plates supplemented with antibiotics appropriate for the selection of recipient [60μg/ml nalidixic acid (Nx)], donor [100μg/ml rifampicin (Rf)] and recombinant [60μg/ml Nx, 100μg/ml kanamycin (Kn)] bacteria, respectively. Control crosses on agar plates were performed as described previously (Heinemann and Ankenbauer, 1993).

The transmission frequency was calculated as the number of recombinants per limiting intracellular parent (Heinemann and Ankenbauer, 1993; Heinemann *et al.*, 1996). Because the number of bacteria that internalize into tissue culture cells can, and did, vary from experiment to experiment, frequencies must be calculated to a common limiting parent for meaningful comparisons between experiments. Generally, fewer donors than recipients were recovered from tissue culture cells, but on occasions the recipient was the limiting parent.

In any conjugation assay, some recombinants can form after transfer of donor and recipient conjugants to the selection plates (Heinemann *et al.*, 1996). The proportion of recombinants formed after plating was measured by mixing lysates of donor-infected cells with lysates of recipient-infected cells on LB agar plates supplemented with Nx and Kn. Extracellular bacteria in the culture medium were concentrated by centrifugation (15,000×g for 3 minutes), rinsed once in PBS, then enumerated by plating on LB agar. Resulting colonies were transferred by replica plating to LB agar supplemented with Nx and Kn to test for recombinant markers (Lederberg and Lederberg, 1952).

All recombinant colonies were transferred by replica plating to LB agar plates supplemented with Rf to test whether they were spontaneously arising Nx-resistant donors.

RESULTS

Plasmids are transmitted between invasive *S. typhimurium* during infection of cultured human cells. Two conjugative plasmids were tested for transmission between bacteria internalized within human cells. The RP4 (IncPα) and F (IncF1) plasmids used express conjugation genes constitutively (Guiney, 1993; Ippen-Ihler, 1986). Moreover, they have interesting differences in their frequencies of transmission in liquid and on solid environments (Bradley *et al.*, 1980). Whereas F plasmids are transmitted at high frequencies in both environments, IncP plasmids transmit between bacteria at much higher frequencies on solid surfaces.

Transmission frequencies of RP4 and F were measured by mating experiments on LB agar plates as previously described (Heinemann and Ankenbauer, 1993) (Table 2). F was found to transmit between the SL1344 strains at only 1% the RP4 frequency (Table 2). This was expected because the *Salmonella* virulence plasmid, pSLT, inhibits F transmission (Sanderson and MacLachlan, 1987). The fertility inhibition caused by pSLT was relieved using the pSLT-cured strain 14028-P (Table 2).

TABLE 2. Agar plate transmission frequencies [a]

Donor (plasmid)[b]	Transmission frequency [c]
SL1344^{R} (RP4)	0.2 ± 0.04
Δ*invA* SL1344^{R} (RP4)	0.2 ± 0.06
SL1344^{R} (pRK21558)	$2 \times 10^{-6} \pm 1 \times 10^{-6}$ [d]
SL1344^{R} (F42::miniTn*10Kn*)	$1 \times 10^{-3} \pm 4 \times 10^{-4}$
14028^{R}-P (F42::miniTn*10Kn*)	3 ± 1
Δ*invA* 14028^{R} (F42::miniTn*10Kn*)	2 ± 1
14028^{R}-P (Jp143)	$4 \times 10^{-6} \pm 9 \times 10^{-7}$ [d]

[a] Bacteria were mixed for two hours on LB agar plates at 37°C.

[b] Recipient *S. typhimurium* were SL1344^{N} and BA770 in RP4 and F matings, respectively.

[c] Number of transconjugants per limiting parent. The frequencies are averages ± standard deviations based on three independent experiments performed in triplicate

[d] The values are maxima. Five of nine replicates (pRK21558) and three of nine replicates (Jp143) produced no detectable transconjugants. The values are averages of values obtained with replicates where transconjugants were detected.

Monolayers of the human intestinal cell line INT-407 were infected with recipient (plasmid-less) *S. typhimurium* and donor *S. typhimurium* carrying either RP4 or F. In order to preclude gene exchanges outside of human cells, cell monolayers were infected in series with an intervening incubation in Gm to kill extracellular bacteria (see Materials and Methods). Donor invasion was halted by the addition of Gm (Isberg and Falkow, 1985). While some cells detached and were washed away during the course of the experiment, the majority remained attached and appeared viable throughout the experimental time course. However, more sensitive assessments of viability were not made.

Recombinant bacteria were recovered on LB selection media after cell lysis by selection for transmission of plasmid-borne antibiotic resistance markers to the recipient bacteria. Recipient bacteria with RP4 markers were recovered at a frequency of 2×10^{-4} recombinant colonies per limiting parent (Table 3). F was transmitted between intracellular *Salmonella* at a frequency of 7×10^{-4} (Table 3). All recombinants scored positive for a second plasmid marker, tetracycline resistance (RP4) or the ability to use lactose as a sole carbon source (F42::miniTn*10Kn*).

TABLE 3. Intracellular transmission by Tra$^+$ and Tra$^-$ plasmids [a]

Expt	Replicate	Transmission frequency			
		RP4	pRK21558	F42::miniTn*10Kn*	Jp143
1	1	2×10^{-4} (142)[b]	$\leq 2 \times 10^{-6}$ (0)	4×10^{-4} (60)	$\leq 1 \times 10^{-5}$ (0)
	2	3×10^{-4} (126)	$\leq 1 \times 10^{-6}$ (0)	7×10^{-4} (63)	$\leq 7 \times 10^{-6}$ (0)
	3	4×10^{-4} (128)	$\leq 2 \times 10^{-6}$ (0)	2×10^{-4} (45)	$\leq 1 \times 10^{-5}$ (0)
2	1	1×10^{-4} (43)	$\leq 2 \times 10^{-6}$ (0)	2×10^{-3} (123)	$\leq 2 \times 10^{-5}$ (0)
	2	1×10^{-4} (84)	$\leq 1 \times 10^{-6}$ (0)	9×10^{-4} (428)	$\leq 1 \times 10^{-6}$ (0)
	3	1×10^{-4} (48)	$\leq 1 \times 10^{-6}$ (0)	1×10^{-3} (335)	$\leq 1 \times 10^{-6}$ (0)
3	1	3×10^{-4} (382)	$\leq 4 \times 10^{-6}$ (0)	2×10^{-4} (272)	$\leq 2 \times 10^{-7}$ (0)
	2	4×10^{-4} (417)	$\leq 2 \times 10^{-5}$ (0)	6×10^{-4} (266)	$\leq 7 \times 10^{-7}$ (0)
	3	3×10^{-4} (523)	$\leq 7 \times 10^{-6}$ (0)	4×10^{-4} (112)	$\leq 4 \times 10^{-7}$ (0)
Average		$2 \times 10^{-4} \pm 6 \times 10^{-5}$ (1893)		$7 \times 10^{-4} \pm 3 \times 10^{-4}$ (1704)	

[a] In IncP experiments the donor was either SL1344^{R} (RP4) or SL1344^{R} (pRK21558) and the recipient was SL1344^{N}. In IncF experiments, the donor was either 14028^{R}-P (F42::miniTn*10Kn*) or 14028^{R}-P (Jp143) and the recipient was BA770. Plasmid transmission frequencies are expressed as the number of transconjugant colonies per limiting intracellular parent.

[b] The numbers in parentheses are the number of recombinant colonies observed, totalled in the final row.

Trivial explanations for the recovery of recombinants were eliminated by control experiments. First, the possibility that the recombinants were instead spontaneous Nx-resistant mutants of donors (the marker used to uniquely select recombinant recipients) was eliminated by confirming that putative recombinants were still sensitive to Rf (the donor-specific marker). Second, the ability for equivalent numbers of bacteria, released from independently infected cells onto selective media, to mate on the enumeration plates was measured (see Materials and Methods) and found not to account for a significant proportion of recombinants. The total number of post-plating recombinant colonies observed in eight independent triplicate experiments amounted to 138 (see Table 1 in the Appendix to Chapter 3). In the same experiments, 9038 bacterial recombinants were recovered from mixed infections of tissue culture cells. Post-plating plasmid transmission thus accounted for an average of 2.3% of recombinants and never contributed greater than 10% of the observed recombinants. No post-plating recombinant colonies were observed in a total of three independent triplicate experiments using F plasmids, whereas1704 recombinants were observed in mixed infections of tissue culture cells (Table 1, Appendix to Chapter 3). Therefore, fewer than 0.06% of these recombinants could be a result of gene transfer occurring after plating bacteria on selection media.

Intracellular recombinants accumulate steadily over time. Intracellular *Salmonella* reside inside vacuoles (Finlay and Cossart, 1997) and have been shown to do so inside cultured cells for at least the length of time relevant to our experiments (Finlay *et al.*, 1991). We therefore predicted that for conjugation to occur, intracellular donor- and recipient-containing vacuoles must either fuse or a small proportion of bacteria must be released into the cytoplasm.

Salmonella-containing vacuoles reportedly coalesce twelve hours after infection, with bacteria observed inside one large perinuclear vacuole (Finlay and Falkow, 1990; Finlay *et al.*, 1991). If vacuole fusion were required for intracellular conjugation, intracellular recombinants might not be detected before 12 hours after

donor internalization. However, we observed intracellular recombinants by three hours after donor invasion (Fig. 1).

The number of intracellular recombinants increased two orders of magnitude over six hours, from our limit of detection frequency of ~10^{-6} (Table 5), to an average frequency of $7 \times 10^{-4} \pm 2 \times 10^{-4}$ recombinants per limiting parent (Fig. 1). This time-dependent increase in recombinant frequency was not due to faster growth of newly formed recombinants relative to parent bacteria. In reconstruction experiments, cells infected by a mixture of donors, recipients and nascent recombinants did not accumulate intracellular recombinant bacteria any faster than they accumulated parental bacteria (Fig. 1, Appendix to Chapter 3). Thus, the increase in frequency was likely due to accumulation of new recombinants.

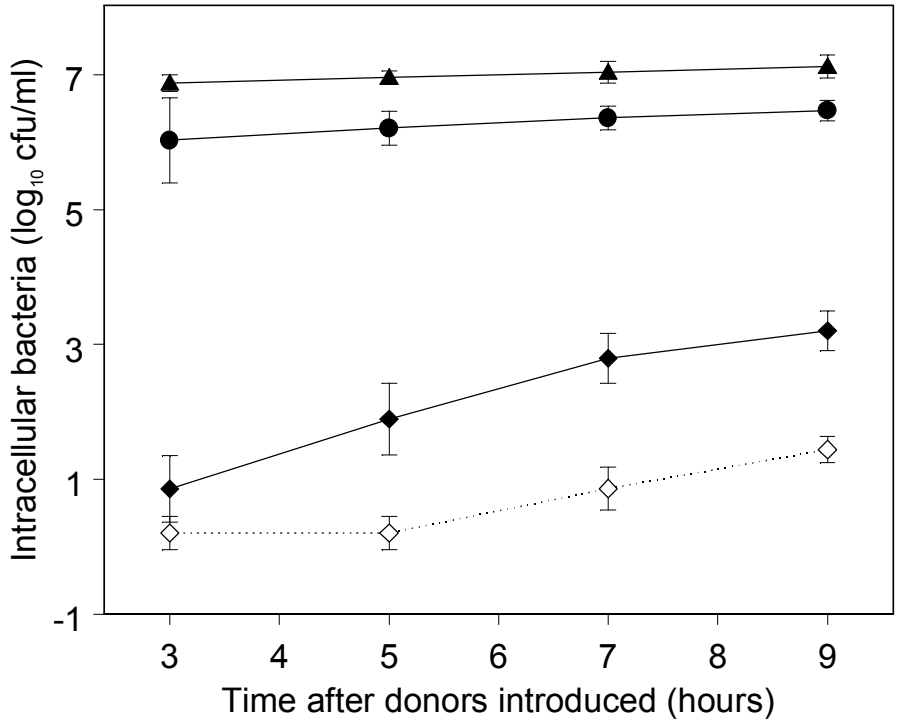

FIG. 1. The accumulation of intracellular transconjugants over time.
Intracellular recipients ($SL1344^N$) (▲), donors [$SL1344^R$ (RP4)] (●) and transconjugants (♦) were enumerated over time, beginning six hours after recipient invasion began (or 2.75 hours after donor invasion began). The number of transconjugants formed after plating (◊) was not significantly different at the first time point. Each value is an average based on three independent experiments performed in triplicate. Error bars indicate standard errors.

Gene exchange does not occur extracellularly. Some proportion of extracellular recipient bacteria may have escaped Gm killing or emerged from cells and mated with extracellular donors during the time donor bacteria were infecting cells. If this were the source of the recombinants, then they should be created even if the donor bacteria could not invade. Therefore, the intracellular gene transfer experiment, described above with invasive donors, was repeated using the non-invasive donors Δ*invA* SL1344^{R} and Δ*invA* 14028^{R}-P, which are compromised for invasion by Tn*PhoA* insertions in the *invA* gene (Galán and Curtiss III, 1989). InvA is an inner membrane protein that may form part of the Type III Secretion System (Galán *et al.*, 1992). Although *invA* mutants are capable of binding to the cell surface, they invade at least 100× less frequently than wild-type bacteria (Table 2, Appendix to Chapter 3) (Galán and Curtiss III, 1989).

Recombinants were rarely and inconsistently detected in the intracellular mating assay when donors were non-invasive (Table 4), even though *invA* and wild-type donors transmit RP4 and F at comparable frequencies to recipient *S. typhimurium* mixed on LB agar plates (Table 2).

No recombinants containing RP4 were recovered using *invA* mutant donors whereas a small number of recombinants containing F were recovered. The difference reflects a differential effect of the *invA* mutation on the invasion efficiency of SL1344 and of 14028. Intracellular Δ*invA* 14028^{R}-P (F42::miniTn*10Kn*) bacteria were recovered at an average of 3% of the number of inv^{+} bacteria whereas intracellular Δ*invA* SL1344^{R} (RP4) bacteria were recovered at an average of 0.1% of the number of inv^{+} bacteria. It appears that a threshold of 10^{4} intracellular donors is required to detect the formation of recombinants by this assay..

TABLE 4. Intracellular plasmid transmission requires donors to be invasive [a]

Expt	Replicate	Transmission frequency			
		Invasive donors (RP4)	Non-invasive donors (RP4)	Invasive donors (F42::miniTn*10Kn*)	Non-invasive donors (F42::miniTn*10Kn*)
1	1	8×10^{-5} (142)[b]	$\leq 3 \times 10^{-7}$ (0)	2×10^{-5} (60)	$\leq 4 \times 10^{-7}$ (0)
	2	7×10^{-5} (126)	$\leq 3 \times 10^{-7}$ (0)	3×10^{-5} (63)	8×10^{-7} (2)
	3	7×10^{-5} (128)	$\leq 3 \times 10^{-7}$ (0)	2×10^{-5} (45)	8×10^{-7} (2)
2	1	2×10^{-5} (43)	$\leq 8 \times 10^{-8}$ (0)	2×10^{-5} (123)	4×10^{-7} (4)
	2	4×10^{-5} (84)	$\leq 8 \times 10^{-8}$ (0)	8×10^{-5} (428)	1×10^{-7} (1)
	3	2×10^{-5} (48)	$\leq 8 \times 10^{-8}$ (0)	8×10^{-5} (335)	2×10^{-7} (2)
3	1	3×10^{-5} (382)	$\leq 5 \times 10^{-8}$ (0)	2×10^{-5} (272)	$\leq 2 \times 10^{-7}$ (0)
	2	3×10^{-5} (417)	$\leq 5 \times 10^{-8}$ (0)	3×10^{-5} (266)	$\leq 2 \times 10^{-7}$ (0)
	3	3×10^{-5} (523)	$\leq 5 \times 10^{-8}$ (0)	2×10^{-5} (112)	$\leq 2 \times 10^{-7}$ (0)
Average		$4 \times 10^{-5} \pm 1 \times 10^{-5}$ (1893)	(0)	$4 \times 10^{-5} \pm 1 \times 10^{-5}$ (1704)	(11)

[a] In RP4 experiments, the donor was either SL1344^{R} or Δ*invA* SL1344^{R} and the recipient was SL1344^{N}. In F experiments, the donor was either 14028^{R}-P or Δ*invA* 14028^{R}-P and the recipient was BA770. Plasmid transmission frequencies are expressed as the number of transconjugant colonies per input donor.

[b] The numbers in parentheses are the number of recombinant colonies observed.

In addition to the control experiment with non-invasive donors we repeated the above experiment with donors that had been incubated in 100μg/ml gentamicin for one hour prior to mixing with tissue culture cells. Efficient killing of the bacteria by gentamicin was confirmed by plate counts. No recombinants were recovered when donors were non-viable, therefore the observed recombinants in experiments with viable bacteria are not due to conjugation between dead donors and viable recipients in the cell culture medium (data not shown). Further, gentamicin-mediated killing was shown to be an extremely effective inhibitor of conjugation in mating experiments carried out between bacteria mixed on LB agar plates (data not shown).

The mechanism of plasmid transmission is conjugation. The formal possibility remained that plasmids, released from lysed internalized donors, transformed competent internalized recipients. Transformation is an unlikely mechanism of transmission because *Salmonella* is not naturally competent for DNA uptake. Nevertheless, the vacuole might induce competence in unknown ways.

If the mechanism of transmission were conjugation, it should be sensitive to mutations in the *tra* (transfer) genes of the plasmids. To distinguish decisively between conjugation and transformation as the mechanism of RP4 transmission, a Δ*traJ* derivative of RP4 (Sia *et al.*, 1995) and a *traB* derivative of F (Heinemann *et al.*, 1996) were introduced into the donor strains. TraJ forms part of the relaxosome complex required for nicking and unwinding of single-stranded plasmid DNA (Grahn *et al.*, 2000). *traB* is necessary for conjugative pilus formation (Ippen-Ihler, 1986). The *traJ* deletion significantly reduced plasmid transmission on agar plates (Table 2). Likewise, *traJ* was necessary for the formation of intracellular recombinants (Table 3). Similarly, F plasmids with mutations in *traB* produced no intracellular recombinants (Table 3).

In addition, if transformation were contributing to the formation of recombinants, the effect should be enhanced with smaller plasmids. However, no recombinants were detected when donor bacteria carried either the 4.4Kb plasmid pBR322 or the

10.5Kb plasmid pSUP104 (Priefer *et al.*, 1985) (data not shown). Conjugation and not transformation is therefore the likely mechanism of the intracellular plasmid transmission measured in these experiments.

Transconjugants do not form from conjugation on the well surface beneath the monolayer. Since *Salmonella* has been shown to traverse cell monolayers (Finlay *et al.*, 1989; Finlay and Falkow, 1990), it was possible that conjugation was occurring on the well surface beneath the cells in 'pockets' not penetrated by Gm-containing medium. To control for this possibility, we performed two experiments. In the first, tissue culture cells were seeded onto permeable membrane supports (see Materials and Methods) inserted inside wells. This allowed both sides of the monolayer to be bathed in Gm, thus exposing any potential bacterial escapees. Transconjugants were recovered at a frequency indistinguishable from that where cells were grown on a plastic surface (Table 5).

TABLE 5. Plasmid transmission frequencies within adherent and non-adherent cells [a]

INT-407 cells	Total number of recombinants recovered [b]	Transmission frequency [c]
Monolayer on permeable filter support, co-infected[d]	338 (1260)	2×10^{-4} (3×10^{-4})
Nonadherent, co-infected[e]	4475 (5278)	2×10^{-4} (3×10^{-4})
Nonadherent, mixed after independent infection[f]	22 (1406)	2×10^{-6} (3×10^{-4})
Recombinants formed after plating	22 (1406)	9×10^{-6} (3×10^{-4})

[a] The donor and recipient bacteria were $SL1344^{R}$ (RP4) and $SL1344^{N}$, respectively.

[b] The numbers in parentheses are the numbers of recombinant colonies recovered in the corresponding positive control experiments [INT-407 monolayers infected in series with $SL1344^{N}$ and $SL1344^{R}$ (RP4)].

[c] In most cases plasmid transmission frequencies are expressed as the total number of transconjugants per limiting parent as determined in three representative experiments performed in triplicate; two experiments were performed in triplicate with the co-infected monolayers on permeable filter supports. The plasmid transmission frequencies in parentheses are the frequencies from the corresponding positive control experiments.

[d] 'Co-infected' refers to cells that were infected with both recipient and donor bacteria.

[e] 'Non-adherent' cells were monolayers treated with trypsin after the second Gm-bathing period.

[f] Independent infection' indicates cells separately infected with either donors or recipients.

Many fewer transconjugants were recovered in experiments using membranes. Since the surface area of an insert membrane was approximately half that of a well, bacteria released from cells grown on two inserts were pooled to make one replicate. Despite this, recovery of intracellular bacteria was poor, a result of both poor adhesion of cells to the membrane and reduced bacterial invasion efficiency. In a second experiment, cells were released from the monolayer by treatment with trypsin after the second Gm-bathing period (0.25% trypsin; 1mM EDTA, Gibco) and re-seeded into wells as a cell suspension in Gm-containing medium. No significant cell re-adherence was observed during the experiment. Again, transconjugants were detected at a frequency and number indistinguishable from that observed with undisturbed monolayers (Table 5).

As a further control for extracellular mating, cells were infected with either recipient or donor bacteria and were then released from the monolayer with trypsin. Infected cells were then mixed and seeded in a clean well. Transconjugants were only detected at the frequency at which they formed after plating of the lysate (Table 5). Together, these experiments confirm that transconjugants were not formed by extracellular conjugation between emergent bacteria and suggest that donors and recipients must concurrently infect the same cells in order for transconjugants to form.

The frequency of transconjugants decreases with decreasing likelihood of co-infection. For intracellular conjugation to occur, donor and recipient bacteria must make contact inside cells. Consistent with this fact, the frequency of transconjugants was found to decrease with a decreasing ratio of invading bacteria to cells (the MOI) which decreases the probability of donor and recipient concurrently occupying the same cell (Fig. 2A).

Plasmid transmission was measured in experiments conducted over a range of MOIs from 0.1 to 100. In each experiment, the same number of donors as recipients was introduced to cell culture. Thus, the total number of bacteria to

tissue culture cells is always twice the MOI reported. The number of recipients internalized after three hours was determined by lysis and plating of bacteria from replicate wells directly after the Gm-bathing period. The initial number of intracellular recipients increased with MOI. MOIs above 10 had no additional effect on the number of internalized bacteria (Fig. 2B).

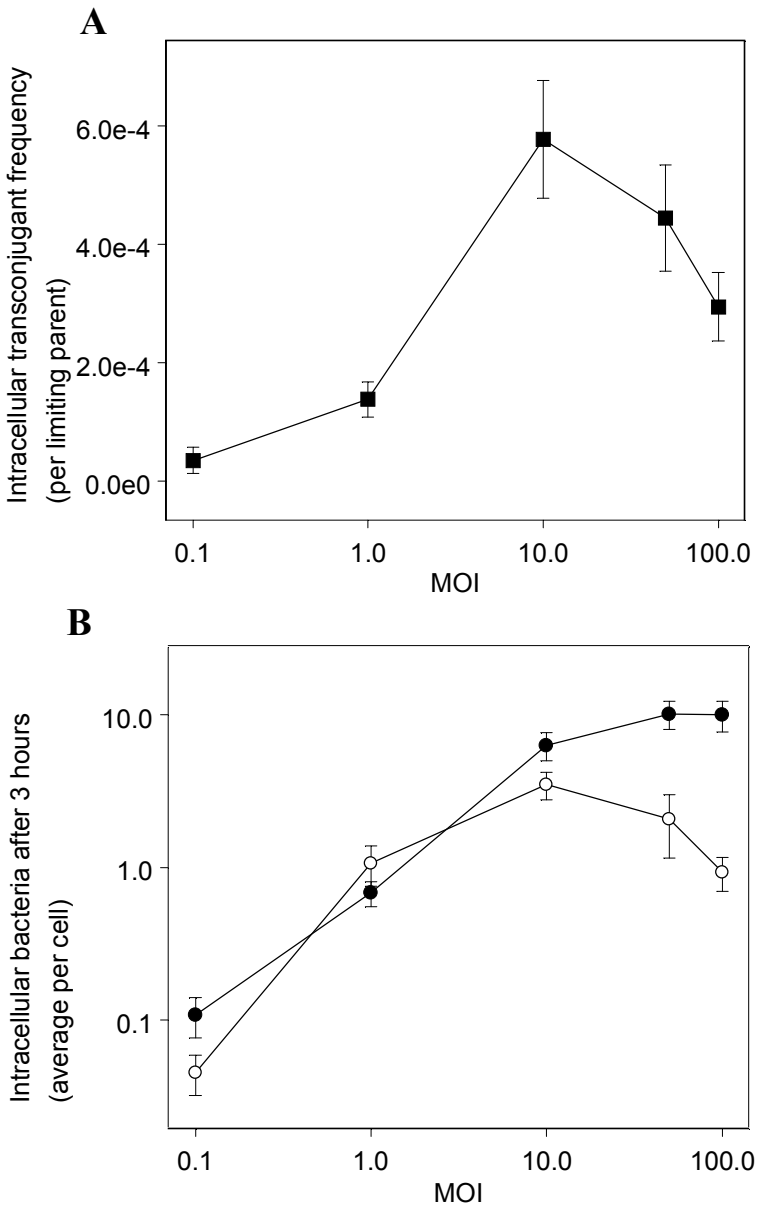

FIG. 2. The intracellular conjugation frequency increases with the probability of co-infection. (A) Transconjugant frequency measured as a function of MOI. (B) Invasion of tissue culture cells by recipients (●) and donors (○) measured as a function of MOI. Each value is an average based on three independent experiments performed in triplicate.

The observed plateau in the number of internalized bacteria is consistent with reported observations that *Salmonella* invasion kinetics reach saturation at MOIs above 40. Saturation is attributed to a limited number of binding sites on the cell surface (Huang *et al.*, 1998; Kusters *et al.*, 1993). The initial number of donors that invaded cells pre-infected with recipients at each MOI was therefore also measured. As expected, fewer donors invaded cells pre-infected with a high number of recipients (Fig. 2B). Again, this is likely due to the internalization of binding sites during the first infection (Huang *et al.*, 1998).

The frequency of transconjugants (Fig. 2A) is proportional to the frequency of co-infection (Fig. 2B). The transconjugant frequency increased from $3 \times 10^{-5} \pm 2 \times 10^{-5}$ (where detectable), at an MOI of 0.1, to $6 \times 10^{-4} \pm 1 \times 10^{-4}$, at an MOI of 10. The peak in frequency at an MOI of 10 corresponded to the highest number of both donors and recipients internalized. Therefore, the intracellular conjugation frequency was dependent on the probability of co-infection. The frequency of transconjugants was lower at MOIs above 10 for unknown reasons, but possibly because cells infected by large numbers of bacteria became more permeable to Gm or were otherwise lost due to the cytotoxicity associated with heavy infection.

DISCUSSION

Salmonella transferred plasmids within cultured INT-407 human cells. Mating was detected at frequencies in excess of 10^{-4} per limiting parent for RP4 and F. This frequency is only 3 orders of magnitude below that detected on agar plates at much higher concentrations of bacteria. The INT-407 cell line we used was not a special case because transconjugants also formed in dog kidney (MDCK) and human colon cancer cells (CaCo-2) (data not shown). These observations support suggestions that antibiotic resistance genes can, and do, transfer within humans and animals (Prodinger *et al.*, 1996; Summers *et al.*, 1993).

Gene transmission between bacteria occurs inside animal cells by conjugation. The requirement that donors be invasive, that both parental types occupy the same cell concurrently, and that transconjugants remain inside the cell at all times prior to harvest, demonstrates that gene transmission was intracellular.

The dependence on *tra* genes for the formation of recombinants demonstrates that gene transmission was by conjugation and not by transformation. The detection limits of these experiments varied from a high of ~10^{-5} to a low of ~10^{-7} (Table 3). This variation is attributed to variation in internalization of bacteria from experiment to experiment. In some replicates fewer than average intracellular bacteria were recovered, reducing the sensitivity of the experiment. However, across three replicate experiments, performed in triplicate, no recombinant colonies were recovered when donors carried *traJ* or *traB* plasmids compared with a total of 1893 and1704 recombinant colonies for Tra^+ RP4 and F, respectively.

What is the frequency of intracellular gene transmission between invasive pathogenic bacteria? The overall frequency of transmission of ~10^{-4} is the product of (i) how often donors and recipients infect the same cell, (ii) how often the bacteria could form contacts within the cells, and (iii) the frequency of plasmid

transmission between bacteria in contact. Possibly, dual infection of single human cells by both donor and recipient bacteria is common and the frequency is a true reflection of gene transmission efficiency. Alternatively, conjugation within human cells may be as efficient as on agar plates but donors and recipients are less often in direct contact because the co-infection of human cells is infrequent or contact between bacteria inside human cells is rare. This question is addressed further in the following chapter.

Our experiment required donor and recipient bacteria, initially in separate vacuoles, to somehow meet despite that fact that *Salmonella* is known to remain inside vacuoles for extended periods. That the mechanism of gene transfer was found to be conjugation rather than transformation suggests that they do meet, because conjugation requires bacteria-bacteria contact. We propose two models to explain how the bacteria find one another. In the first model, we imagine a small proportion of intracellular bacteria of each parental type escape the vacuole. Transconjugants are subsequently formed by conjugation in the cytoplasm. In the second model, we imagine that transconjugants are formed after fusion of donor- and recipient-containing vacuoles, which is known to occur within 12 to 24 hours (Finlay and Falkow, 1990; Finlay *et al.*, 1991).

A very recent study reports a low level of *S. typhimurium* escape from vacuoles (Brumell *et al.*, 2002). This phenomenon appears to be due to failure of a proportion of *S. typhimurium* to maintain the integrity of the vacuolar membrane rather than an active process of vacuole destruction. If conjugation were occurring outside of vacuoles, then it might involve this small proportion of cytoplasmic *S. typhimurium*. The vacuole fusion model is supported only if vacuole coalescence occurs, at least at some level, before the reported 12 hours. To our knowledge, the time-course of vacuole coalescence has not been investigated. This question is also addressed in the following chapter.

Of what relevance are these observations to plasmid and bacterial evolution?
Bacterial conjugation is probably the primary mechanism of antibiotic resistance-gene transmission (Heinemann, 1999; Mazel and Davies, 1999). The ubiquity of plasmid-borne resistance may in part be explained by the stamina of conjugation itself (Heinemann, 1999). Conjugation can occur even in environments that otherwise "kill" the bacterium, allowing plasmids to replicate by horizontal gene transfer in the presence of antibiotics and other environmental toxins that prevent bacterial reproduction (Cooper and Heinemann, 2000b; Heinemann and Ankenbauer, 1993; Heinemann, 1999).

Plasmids carry a range of genes in addition to resistance determinants, including genes that accentuate virulence and symbiosis potential in many different microbes (Heinemann *et al.*, 2000), from soil (Ankenbauer and Nester, 1993) to human flora (Hueck, 1998; Katayama *et al.*, 1996; Bacon *et al.*, 2000; Cheetham and Katz, 1995). What is the mechanism creating linkage between various catabolic pathways, virulence and resistance genes (Heinemann *et al.*, 2000)? Here we have considered the possibility that, in at least one environment, gene transfer concentrates virulence and resistance determinants, providing opportunity for the evolution of genetic linkage. When the invasive form of *Salmonella* is inside human cells, it can exchange plasmids with other strains. Since virulence is in part dependent upon invasion, the most virulent bacteria will likely be inside cells more often than less virulent bacteria. The most virulent pathogens are also more likely to attract the attention of antibiotic-dispensing clinicians who effectively remove other microbes from the extracellular niche and concentrate resistant microbes in the patient (van der Waaij *et al.*, 1971). Mammalian cells infected by *Salmonella* can subsequently internalize other species of bacteria (Francis *et al.*, 1993), which, in patients treated with antibiotics, must be antibiotic resistant. These other species may then transfer antibiotic resistance genes to *Salmonella*.

Further, it is known that antibiotic treatment causes overgrowth of resistant microflora, increasing translocation of these from the gut to the mesenteric lymph

nodes (Berg, 1981). Translocation most likely occurs by intracellular passage of bacteria through the intestinal epithelium (Berg, 1995). Thus, antibiotic usage may increase the potential for intracellular gene exchange, giving the pathogen and less virulent strains the opportunity to acquire the genes that confer antibiotic resistance and virulence characters. The phenomenon of *in situ* mating provides an evolutionary mechanism for the co-evolution of resistance and novel virulence traits.

Tissue culture experiments establish the plausibility of gene transmission within humans and animals, particularly within their cells, but do not establish the process as relevant to evolution. The frequency of co-infection, intracellular contact between donors and recipients and expression of conjugative functions may differ in the gut environment. These experiments do, however, directly attest to the plausibility of gene transmission during antibiotic therapy. It is interesting to note that gene transfer appears to have occurred historically between the obligate intracellular pathogens *Chlamydia trachomatis and Rickettsia prowazekii*, possibly by a mechanism similar to that described here (Wolf *et al.*, 1999).

Horizontal gene transfer is a surprisingly robust and common phenomenon, defying the most concerted efforts to control microbial diseases (Heinemann, 1999; Heinemann *et al.*, 2000; Heinemann and Roughan, 2000). Understanding the evolution of gene transfer will be as relevant to constructing a lasting strategy to control infectious diseases as is mining the genome for new drug targets or restricting the application of antibiotics.

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Chapter 4: Investigating the Mechanics of Bacterial Conjugation within Cultured Epithelial Cells

ABSTRACT

The experiments presented in this chapter are preliminary attempts to identify the location and distribution of transconjugants within co-infected mammalian cells. Two novel strategies – a plating assay and a fluorescence-based gene transfer assay for the specific detection of intracellular transconjugants – were developed in order to determine the intracellular *distribution* of transconjugants. The *location* of intracellular transconjugants was tested by intracellular conjugation experiments using bacteria mutated or genetically engineered to escape the vacuole into the cytoplasm. It was predicted that relocation of one parental strain to the cytoplasm should result in an *increased* frequency of intracellular conjugation if intracellular conjugation occurred predominantly in the cytoplasm. Conversely, the frequency of conjugation should be *decreased* if intracellular conjugation occurred predominantly within coalesced donor- and recipient-containing vacuoles. Although firm conclusions are not possible due to a number of technical difficulties, the experiments illustrate the limits and value of various experimental methods for characterizing the mechanistic particulars of intracellular conjugation.

INTRODUCTION

How do intracellular conjugants meet?

In order to convincingly demonstrate that plasmid transmission occurred *within* cells, the intracellular conjugation assay performed in Chapter 3 was designed to stringently preclude extracellular mating. Because donor and recipient bacteria were introduced to cultured cells sequentially, preventing their initial uptake into the same vacuoles, the stringency of the experiments emphasizes the minimum frequency of conjugation, which might be much higher. Since conjugation requires physical contact between donors and recipients, mechanistic models for intracellular conjugation must provide for conjugants to meet within cultured cells. Two models with this provision were proposed: (1) the 'vacuole escape model' (Fig.1, A), where a small proportion of *S. typhimurium* are released from vacuoles and conjugate in the cytoplasm, and (2) the 'vacuole coalescence model' (Fig.1, B), where conjugation occurs within donor- and recipient-containing vacuoles that have fused.

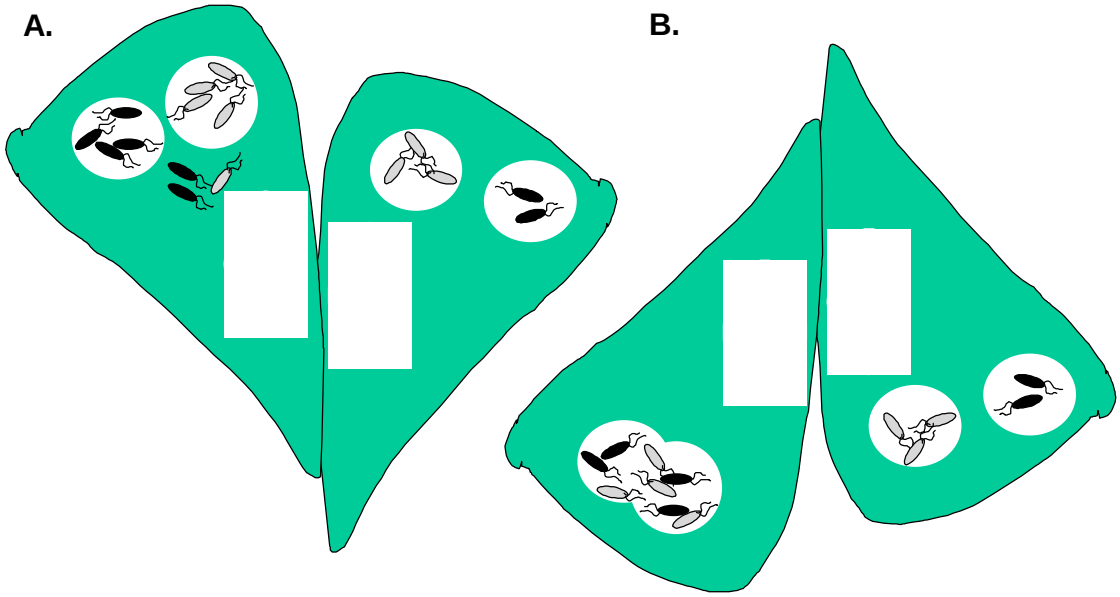


FIG.1. Models for intracellular conjugation. A. Vacuole escape model. B. Vacuole coalescence model.

There is evidence for both models. Coalescence of *Salmonella*-containing vacuoles (SCVs) into one large perinuclear vacuole per cell was observed 12-24 hours following invasion (Finlay and Falkow, 1990; Finlay *et al.*, 1991). Similarly, small phagosomes containing adherent invasive *Escherichia coli* (AIEC) fused to form a single large phagosome per macrophage 8-24 hours following phagocytosis (Glasser *et al.*, 2001). Some pathogens actively direct coalescence of their containing vacuoles: type I *Helicobacter pylori* induces homotypic fusion of phagosomes to form “megasomes” during the first hours following phagocytosis (Allen *et al.*, 2000). “Megasome” formation required bacterial protein synthesis, functional host cell microtubules and was essential for bacterial survival within macrophages. Conversely, credence is given to the vacuole escape model by the observation that 1-5% of intracellular *S. typhimurium* were no longer associated with a vacuolar membrane marker ten hours following entry into cultured epithelial cells (Brumell *et al.*, 2002).

The vacuole coalescence model makes a unique prediction: if only one conjugant is induced or engineered to escape the vacuole, then plasmid transmission should be prevented by separation of the parental types by a membrane. Conversely, the vacuole escape model predicts that the release of one or both parental types into the cytoplasm increases the potential for conjugation.

Testing the vacuole coalescence model using an S. typhimurium mutant with altered intracellular trafficking.

Intracellular conjugation experiments between *S. typhimurium* and the cytoplasmically-located intracellular pathogen *Shigella flexneri* would appear to be an ideal test system for the vacuole coalescence model. However, the details of the *S. flexneri* intracellular lifestyle differ fundamentally to those of *S. typhimurium* (Galán, 1996; Groisman and Ochman, 1993; Finlay and Cossart, 1997). The mechanistic basis of *S. flexneri* and *S. typhimurium* entry into cultured cells is similar, being mediated by a homologous set of genes and differing only in minor

detail. However, once internalized, *S. flexneri* lyses its containing vacuole and replicates in the cytoplasm. Cytoplasmic *S. flexneri* then spread to adjacent cells by a genetically regulated process dependent on actin polymerization. *S. flexneri* is therefore not a comparative model for putative cytoplasmic *S. typhimurium*[1]. Thus, the vacuole coalescence model was instead tested by engineering *S. typhimurium* with a higher likelihood of vacuolar escape.

A number of methods for genetically engineering *S. typhimurium* vacuolar lysis have been described (Zychlinsky *et al.*, 1994; Gentschev *et al.*, 1995; Osiecki *et al.*, 2001; Brumell *et al.*, 2002; Lutwyche *et al.*, 1998). Interestingly, it appears that maintenance of the vacuolar membrane is determined by *S. typhimurium*, and paradoxically so since failure to maintain the *Salmonella*-containing vacuole (SCV) results in a significant growth advantage within nonphagocytic cells (Stein *et al.*, 1996; Brumell *et al.*, 2001b; Brumell *et al.*, 2002). SCV maintenance is conferred by the product of the *sifA* gene, an SPI-2 secreted effector protein (Hansen-Wester *et al.*, 2002), responsible for the formation of tubular membrane filaments called 'sifs' (*salmonella*-induced filaments). Sifs are rich in lysosomal glyoproteins (lgp) and protrude from SCVs in epithelial cells (Stein *et al.*, 1996). In fact, the formation of sifs within nonphagocytic cells may be an artifact of non-specific induction of the SPI-2 TTS genes (*ssa*/*spi*) in response to the low vacuolar pH (Brumell *et al.*, 2001a). Induction of *ssa*/*spi*, *sifA* and other SPI-2 effector genes is necessary for proliferation of *S. typhimurium* only within murine phagocytic cells, where mutation of these genes results in a significant replication defect (Beuzón *et al.*, 2000).

Hypothetically, growth of *S. typhimurium* within the SCV necessitates an increase in the surface area of the vacuolar membrane (Beuzón *et al.*, 2000). SCV expansion is likely mediated by recruitment of membrane to the SCV by interaction with host cell vesicles (Méresse *et al.*, 1999). Since *sifA* mutants appear in the cytoplasm approximately 5-10 hours following entry into cultured cells (Beuzón *et*

[1] Although intracellular plasmid transmission between *S. typhimurium* and *S. flexneri* would have made an interesting experiment, we lacked the required regulatory approval to perform experiments with *S. flexneri* within our laboratory.

al., 2000; Brumell *et al.*, 2002), SifA may recruit membrane, possibly by stimulation of the GTPase Rab7, a regulator of membrane fusion events (Méresse *et al.*, 1999). Indeed, SifA is secreted out of the SCV and acts at the cytoplasmic face of the vacuolar membrane where it is primely located for such a function (Brumell *et al.*, 2001a; Beuzón *et al.*, 2000). SifA appears to act in concert with at least one other SPI-2 secreted protein, SseJ (Ruiz-Albert *et al.*, 2002), to recruit membrane to the SCV, possibly by mediating fusion with small lysosomal transport vesicles whose normal trafficking may be inhibited by *S. typhimurium* (Méresse *et al.*, 1999).

A *S. typhimurium sifA* mutant was therefore considered an ideal cytoplasmic candidate for test of the vacuolar coalescence model. Incubation of epithelial cells infected with *sifA*$^-$ bacteria for a sufficient period time should result in relocation of a proportion of intracellular bacteria to the cytoplasm (Brumell *et al.*, 2002). Furthermore, the *sifA* mutation appears to amplify an inherent tendency for *S. typhimurium* vacuolar escape. That is, 1-5% of wild-type intracellular *S. typhimurium* exhibited the same characteristics of *sifA* mutants, with loss of vacuolar membrane integrity and the appearance of cytoplasmic bacteria with a significantly increased growth rate. Thus, if intracellular conjugation results from contact between small numbers of bacteria released into the cytoplasm, the frequency of such contact should be enhanced where one or both parents is *sifA*$^-$.

Engineering S. typhimurium strains that rapidly escape the vacuole.

While loss of *sifA* function probably most closely approximates what may occur naturally within a small proportion of wild-type intracellular bacteria, the loss of SCV integrity is likely to affect the intracellular location of only ~50-70% of bacteria over the incubation times relevant to our intracellular conjugation assays (Chapter 3). Thus, a means of generating a higher proportion of cytoplasmic *S. typhimurium* more rapidly was also sought. *S. typhimurium* can be engineered to actively lyse the vacuole using a number of bacterial pore-forming toxins (Lutwyche *et al.*, 1998;

Zychlinsky *et al.*, 1994; Gentschev *et al.*, 1995; Osiecki *et al.*, 2001). Conveniently, the property of vacuole lysis can be conferred upon *S. typhimurium* by substitution of its SPI-1 TTS system effector protein, SipC, with the *S. flexneri* homologue, IpaC (Osiecki *et al.*, 2001). *ipaC* complemented an *S. typhimurium sipC* mutant for invasion with the subsequent release of intracellular S*. typhimurium* into the cytoplasm. Attempts to complement *S. flexneri ipaC* mutants with *sipC* notably failed, in spite of successful secretion of SipC into the culture supernatent by the *S. flexneri* TTS system (*mxi*/*spa*/*ipa*) (Osiecki *et al.*, 2001). This is best explained by the divergent functions of these secreted effectors: IpaC interacts with the host Rho GTPase Cdc42 to initiate the actin rearrangements that lead to internalization [a function carried out in *S. typhimurium* by the SopE protein (Hardt *et al.*, 1998)], whereas SipC interacts with actin to promote nucleation and bundling (Osiecki *et al.*, 2001). Thus, SipC performs a function downstream of the IpaC activity and it is therefore not surprising that SipC cannot complement the loss of IpaC function.

S. typhimurium intracellular conjugation experiments were therefore carried out both with *sifA* mutants and with *ipaC*-complemented *sipC* mutants as recipients in order to test the effect of vacuole escape on the frequency of plasmid transmission.

Determining the true frequency of plasmid transmission between intracellular bacteria.

RP4 transmission between intracellular *S. typhimurium* occurred at a frequency of ~10^{-4} per limiting parent (Chapter 3). However, the overall frequency of intracellular plasmid transmission is a product of three variables: (i) how often donors and recipients infect the same cell, (ii) how often the bacteria form contacts within co-infected cells and (iii) the frequency of plasmid transmission between bacteria in contact. Co-infection of single human cells by both donor and recipient bacteria with subsequent mixing may be common and the overall frequency therefore a true reflection of gene transmission efficiency. This would imply that the intracellular environment is less permissive for conjugative plasmid transmission than the

surface of an LB agar plate (see Chapter 3, Table 2). Alternatively, conjugation within human cells may be as efficient as on agar plates but occurs comparatively infrequently since donors and recipients are less often in direct contact. Again these two possibilities make unique predictions: in the former instance, smaller numbers of transconjugants would be distributed across a larger proportion of co-infected cells. In the latter instance, a larger number of transconjugants would be located within a very small proportion of co-infected cells.

Two experiments were devised to test the intracellular distribution of transconjugants in order to determine the true permissiveness of the intracellular environment for conjugation. The first is a novel plating assay designed to enumerate not the total number of intracellular bacteria, but the proportion of cultured cells infected with each parental and recombinant type. The second is a fluorescence-based genetic assay, designed to exclusively detect intracellular transconjugants by induction of a GFP reporter construct encoded on the incoming plasmid.

MATERIALS AND METHODS

Plasmids and Strains. Strains and plasmids are described in Table 1. pFM10.1::Tc was constructed by ligation of a klenow-blunted *Eco* RI/*Ava* I fragment of pBR322 with pFM10.1 (Valdivia and Falkow, 1997) at the *Sca* I site within the ampicillin resistance gene. Plasmid DNA was prepared for cloning from *E. coli* XL1-Blue. Cloning procedures were carried out according to the standard protocols described by Sambrook *et al.* (Sambrook *et al.*, 1989). Plasmids encoding IncP or IncQ *oriTs* were mobilized to *S. typhimurium* from *E. coli* S17.1-λ pir by conjugation as described previously (Heinemann and Ankenbauer, 1993).

The *S. typhimurium* chromosomal gene *ssrB* was mutated by homologous recombination with a mutated *ssrAB*::Cm allele encoded on the positive-selection allelic-exchange vector $pJQ200_{SK}$ (Quandt and Hynes, 1993). Double crossover events resulted in the loss of a 5' region of *ssrB* and insertion of a chloramphenicol resistance (Cm^R) marker downstream of the deletion. The construction of pGCF13 ($pJQ200_{SK}$::*ssrAB*::Cm) and the procedure for creation of the *ssrB* mutation is outlined in the appendix to this chapter (Appendix to Chapter 4, Figs. 1, 2 and 3). Insertion of the Cm^R gene within *ssrB* was confirmed by PCR and Southern hybridization (Appendix to Chapter 4, Fig. 4). The *S. typhimurium* chromosomal gene *sipC* was deleted using the procedure of Datsenko *et al.* (outlined in Figs. 5, 6 and 7 in the Appendix to Chapter 4) (Datsenko and Wanner, 2000). *sipC* deletion was subsequently confirmed by PCR (Fig. 8, Appendix to Chapter 4).

Mutation of *S. typhimurium* chromosomal genes was carried out within the restriction mutant MS1868. Chromosomal mutations were transferred to wild-type *S. typhimurium* strains by P22 transduction using the high frequency transducing strain $P22_{int3\ HT12/4}$.

TABLE 1. *S*trains and plasmids

Strain or plasmid	Relevant genotype and/or phenotype[a]	Source	Reference
S. typhimurium			
MS1868	LT2 *leuA414* (Am) *hsdR*	I.J. Molineux	
P_3H_6	12023s *sifA*::miniTn*5*; (Kn^r)	D.W. Holden	(Beuzón *et al.*, 2000)
SL1344	Wild-type, *rpsL hisG46*	B.B. Finlay	(Hoiseth and Stocker, 1981)
$SL1344^N$	Spontaneous Nx^r mutant of SL1344	This study	
$SL1344^R$	Spontaneous Rf^r mutant of SL1344	This study	
$SL1344_{\Delta sifA}$	SL1344 Δ*sifA*	B.B. Finlay	(Stein *et al.*, 1996)
$SL1344^N_{\Delta sifA}$	$SL1344^N$ Δ*sifA*	This study	
$SL1344^R_{\Delta sifA}$	$SL1344^R$ Δ*sifA*	This study	
$SL1344^R_{sifA::Kn}$	$SL1344^R$ *sifA*::miniTn*5*; (Kn^r)	This study	
$SL1344^N_{\Delta sipC}$	$SL1344^N$ Δ*sipC*	This study	
SR11	Wild type	B.B. Finlay	(Galán and Curtiss III, 1989)
$SR11^N$	Spontaneous Nx^r mutant of SR11	This study	
$SR11^R$	Spontaneous Rf^r mutant of SR11	This study	
$SR11^R_{ssrB::Cm}$	$SR11^R$*ssrB*::Cm; (Cm^r)	This study	
$SR11^N_{sifA::Kn}$	$SR11^N$ *sifA*::miniTn*5*; (Kn^r)	This study	
$SR11^R_{sifA::Kn}$	$SR11^R$ *sifA*::miniTn*5*; (Kn^r)	This study	
$SR11^N_{\Delta sipC}$	$SR11^N$ Δ*sipC*	This study	
E. coli			
XL1-Blue	*supE44 hsdR17 recA1 endA1 gyrA46 relA1 lac-* F' {*lacI*^{q} *lacZDM15* Tn*10*}; (Tc^r)		(Bullock *et al.*, 1987)

S17.1-λ pir	*hsdR*$^-$ *hsdM*$^+$ Δ*recA* RP4-2-Tc::*Mu*-Kn::Tn*7* λ *pir*; (Sm^r Tp^r)		(Alexeyev and Shokolenko, 1995)
Plasmids			
RP4	IncPα; (Kn^r Ap^r Tc^r)		(Datta *et al.*, 1971)
pRK2526	IncPα; RK2 *tetA*::*lacZYA*; (Kn^r Ap^r)	D. Figurski	(Sia *et al.*, 1995)
pFM10.1	pUC-based replicon encoding *ssaH::gfp*; (Ap^r)	S. Falkow	(Valdivia and Falkow, 1997)
pFM10.1::Tc	pFM10.1 *bla*::*tetA*; (Tc^r, Ap^s)	This study	
pGEM-Teasy	pUC-based PCR cloning vector; (Ap^r)	Promega	
pGEM-Teasy::*ssrA*	*ssrA* PCR fragment ligated with pGEM-Teasy; (Ap^r)	This study	
pGEM-Teasy::*ssrB*	*ssrA* PCR fragment ligated with pGEM-Teasy; (Ap^r)	This study	
pGCF11	pGEM-Teasy::*ssrAB*; (Ap^r)	This study	
pHP45Ω-Cm	pBR322-based replicon encoding an excisable Cm^r cassette; (Cm^r, Ap^r)		(Fellay *et al.*, 1987)
pGCF12	pGEM-Teasy::*ssrAB*::Cm; (Ap^r, Cm^r)	This study	
pJQ200$_{SK}$	*sacB*; oriR*g* (λ *pir*-dependent replication); *oriT*$^+$ *mob*$^+$; (Gm^r)		(Quandt and Hynes, 1993)
pGCF13	pJQ200$_{SK}$::*ssrAB*::Cm; (Cm^r, Gm^r)	This study	
pMLZ205	pACYC184 *tetA*::*phoP::gfp*; (Tc^s, Cm^r)	B.B. Finlay	(Brumell *et al.*, 2002)
pWPsfc	*ipaC*$^+$ (expressed from P_{lac}); pUC *oriV*; (Ap^r)	W.L. Picking	(Osiecki *et al.*, 2001)
pWPsfsipC	*sipC*$^+$ (expressed from P_{lac}); pUC *oriV*; (Ap^r)	W.L. Picking	(Osiecki *et al.*, 2001)
pKD13	Kn^r gene flanked by λ FRT recombination sites; *oriRg* ; (Kn^r, Ap^r)	B.L. Wanner	(Datsenko and Wanner, 2000)
pKD46	Encodes the λ recombination genes *g*, *b* and *exo*; *oriRg* ; (Ap^r)	B.L. Wanner	(Datsenko and Wanner, 2000)
pCP20	Encodes the λ FLP recombinase; *oriRg* ; (Cm^r, Ap^r)	B.L. Wanner	(Datsenko and Wanner, 2000)

[a] Nx^r, naladixic acid resistance; Rf^r, rifampicin resistance; Kn^r, kanamycin resistance; Ap^r, ampicillin resistance; Tc^r, tetracycline resistance; Cm^r, chloramphenicol resistance; Gm^r, gentamicin resistance; Tp^r, trimethoprim resistance; *sacB*, sucrase.

Cell Culture. Human intestinal-407 (INT-407) cells (ATCC CCL 6) and MDCK (Madin Darby Canine Kidney) cells were cultured in 25cm^2 flasks (Nunc) in Minimal Essential Medium (MEM, Gibco) supplemented with 2mM L-glutamine, 2mM non-essential amino acids (Gibco) and 10% Fetal Bovine Serum (FBS, Gibco) and maintained at 37°C in a 10% CO_2 atmosphere. Cells used in these experiments were between passage 5 and 25.

Intracellular conjugation experiments. Bacterial invasion and intracellular conjugation experiments were carried out as described previously (see Materials and Methods, Chapter 3) unless specified otherwise in text.

As in Chapter 3, intracellular bacteria released from lysed cells were enumerated by serial dilution in PBS and plating on LB (Gibco) agar plates supplemented with antibiotics appropriate for the selection of recipient [60μg/ml nalidixic acid (Nx)], donor [100μg/ml rifampicin (Rf)] and recombinant [60μg/ml Nx, and either 100μg/ml kanamycin (Kn) or 20μg/ml tetracycline (Tc)] bacteria, respectively. Control crosses on agar plates were performed as described previously (Heinemann and Ankenbauer, 1993). Unless indicated otherwise, plasmid transmission frequencies were calculated as described in Chapter 3.

Immobilization of unlysed INT-407 cells within LB agar. INT-407 cells were infected with *S. typhimurium* as described previously (Chapter 3). Infected cells were dissociated from the plastic culture tray surface with trypsin (0.25% trypsin; 1mM EDTA, Gibco). Excess trypsin was removed immediately by aspiration and the cells were incubated at room temperature until detachment was observed. Trypsinized cells were resuspended in 0.5ml (per well) of MEM supplemented with 1% FBS and diluted serially in MEM (1% FBS) using a 1ml pipette (Gilson) fitted with a wide bore plastic tip ('blue tip', Invitrogen). Suspended cells were transferred to 55mm petrie dishes (Sarstedt) and mixed with molten LB agar (0.7%) supplemented with antibiotics appropriate for selection of donor (Kn), recipient (Nx) and transconjugant (Nx Kn) bacteria, respectively. Plates were incubated at 37°C overnight until bacterial colonies

were observed. Cell-associated colonies were visualized under 200-400× magnification on a Nikon inverted microscope. Images were captured with a CoolSNAP-Pro™ Digital Camera kit (1392 × 1040 pixels, SciTech) and processed using Adobe Photoshop 6.0 (Adobe Systems).

Fluorescence-Activated Cell Sorting (FACS). FACS analysis of INT-407 cells infected with green fluorescent (GFP) *S. typhimurium* was carried out on a FACS Vantage flow cytometer (Becton Dickinson, Dept. of Pathology, Christchurch School of Medicine). INT-407 cells infected with *S. typhimurium* were dissociated from the plastic culture tray surface with trypsin as described above and resuspended in PBS supplemented with 0.9% FBS at a concentration of ~10^6 cells/ml.

Fluorescence microscopy. INT-407 and MDCK cells were cultured on 13mm glass cover slips placed inside 24-well culture trays. Infected cells were fixed with periodate-lysine-paraformaldehyde (PLP) and permeabilized with methanol according to the protocol of Swanson and Isberg (Swanson and Isberg, 1996). Fixed cells were stained with 0.1µg/ml 4', 6-diamidino-2-phenylindole (DAPI, Sigma) for 5 minutes (Swanson and Isberg, 1996), washed 3 times with PBS then mounted on a glass slide with 2.5µl of 50% glycerol::PBS and fixed with clear nail varnish. Fixed cells were screened for fluorescent intracellular bacteria at 400-1000× magnification on an Olympus BH2-RFCA epifluorescence microscope fitted with a FITC filter set (O515IF excitation filter, BP495 barrier filter with an EY475 supplementary barrier filter) for visualization of GFP fluorescence. DAPI-stained bacteria and cell nuclei were visualized with a UV filter set (L420 excitation filter, UG-1 barrier filter). Images were captured with a CoolSNAP-Pro™ Digital Camera kit (1392 × 1040 pixels, SciTech) and processed using Adobe Photoshop 6.0 (Adobe Systems).

RESULTS

Estimating the distribution of transconjugants within INT-407 cells. Two experiments were designed in order to determine whether the transconjugant colonies, recovered after lysis of cells infected with donor and recipient *S. typhimurium*, resulted from extensive plasmid dissemination within a very small proportion of co-infected cells or from less frequent plasmid dissemination within a larger number of co-infected cells. In the first experiment, infected INT-407 cells were immobilized, unlysed, within LB agar (LBA) supplemented with antibiotics for the respective selection of donor, recipient and transconjugant colony growth within each infected cell. This experiment allowed estimates of the proportion of INT-407 cells harboring donors, recipients and transconjugants to be calculated. In the second experiment, a fluorescence-based genetic assay was designed for the specific detection of intracellular transconjugants by GFP expression.

Immobilization of infected cells within a selective bacterial growth medium. INT-407 cells infected with donor and/or recipient *S. typhimurium* were immobilized, prior to lysis, within a solid bacterial growth medium. Each infected cell could be associated with, at most, a single bacterial colony arising later. It was initially expected that immobilization of infected cells would result in growth of a 'cell-associated' bacterial colony only when infected cells were lysed, either spontaneously or upon the addition of detergents such as SDS or deoxycholate to the medium. Surprisingly, cell lysis appeared to be unnecessary for the appearance of 'cell-associated colonies' since the addition of SDS or deoxycholate did not affect the number or appearance of the bacterial colonies (data not shown). Incubating infected cells within LBA overnight at 37°C produced two kinds of colonies: large colonies that were typical of *S. typhimurium* colonies in appearance, and small colonies that were spherical or football-shaped (Fig. 2, A1 and A2).

Upon closer examination (under a Nikon inverted microscope), a distinction could be made between colonies associated with cells that had lysed and those that

appeared to be contained within unlysed (or not visibly lysed) cells. The majority of cell-associated colonies displayed smooth edges with no discernable bacterial rods (Fig. 2, B1-B4). However, emanation of bacteria from a small proportion of cell-associated colonies was evident (although photographically this was captured merely as haziness surrounding the cell-associated colony) (Fig. 2, D1-D4). The majority of cell-associated colonies may therefore result from extensive bacterial replication within a containing structure. Cell-associated colonies were considerably larger than uninfected immobilized cells (Fig. 2, C1 and C2). What structure may be containing the bacterial colony is unknown.

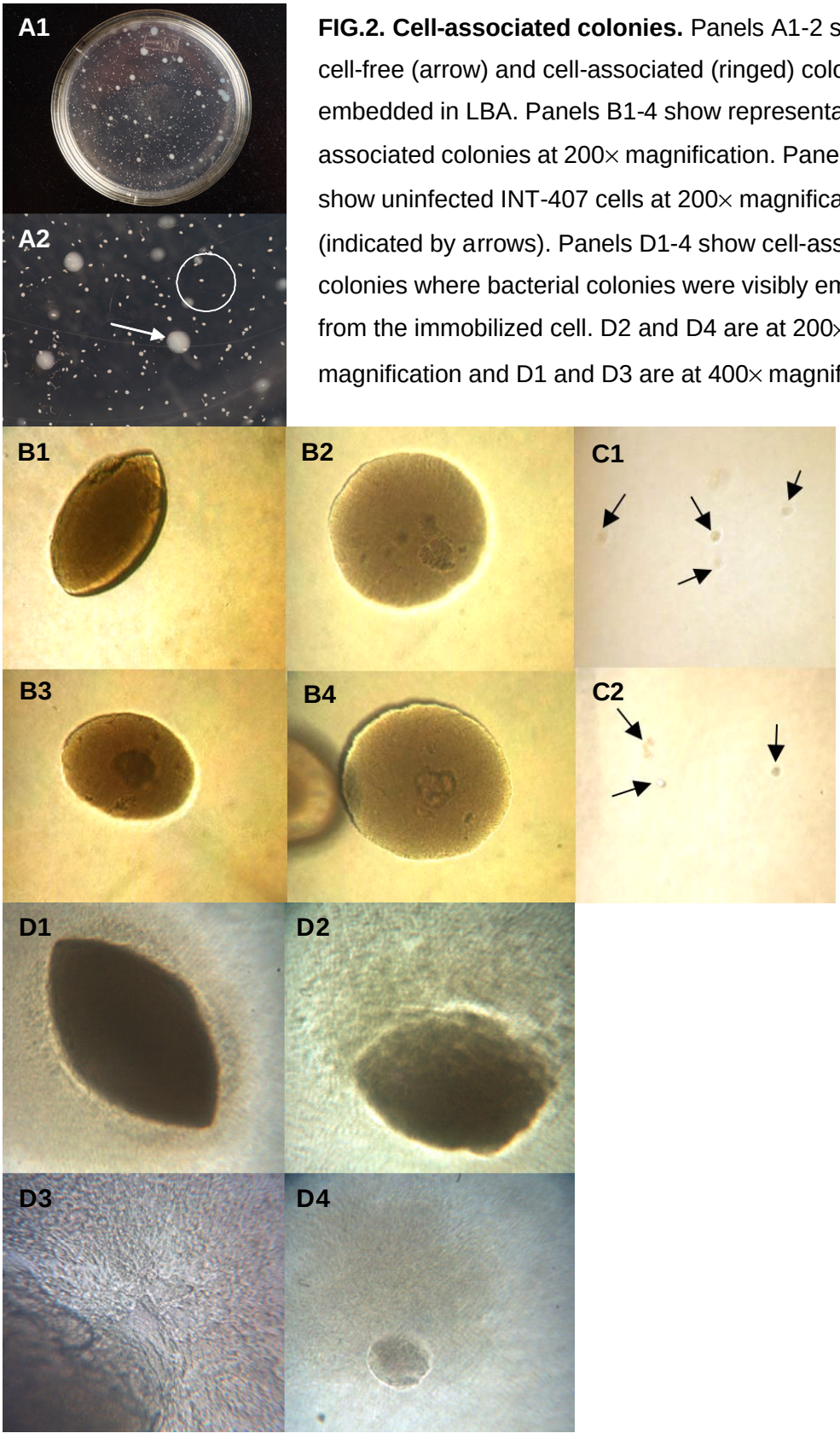


FIG.2. Cell-associated colonies. Panels A1-2 show cell-free (arrow) and cell-associated (ringed) colonies embedded in LBA. Panels B1-4 show representative cell-associated colonies at 200× magnification. Panels C1-2 show uninfected INT-407 cells at 200× magnification (indicated by arrows). Panels D1-4 show cell-associated colonies where bacterial colonies were visibly emanating from the immobilized cell. D2 and D4 are at 200× magnification and D1 and D3 are at 400× magnification.

If nutrients can permeate the immobilized cells to fuel bacterial colony growth, then antibiotics too may penetrate immobilized cells and act selectively upon bacteria. In test of this, cells infected with either donors or recipients were overlaid with LBA supplemented with either Kn or Nx and the appearance of bacterial colonies scored (Table 2). Kn prevented recipient bacteria from forming cell-associated colonies and permitted donor bacteria to form cell-associated colonies. Conversely, Nx prevented cell-associated colony formation by donors and permitted that by recipients. No cell-associated colonies were observed when uninfected cells were immobilized in LBA.

TABLE 2. Growth of bacteria inside unlysed, immobilized cells[a]

	Cell-associated colonies?			
Immobilized cells	No selection	+ Nx	+Kn	+Nx Kn
Uninfected	x	ND	ND	ND
Donor-infected	✓	x	✓	x
Recipient-infected	✓	✓	x	x
Sequential infection with recipients then donors	ND[b]	ND	ND	✓
Donor-infected cells and recipient-infected cells mixed at the time of plating	ND	ND	ND	x

[a]Infected INT-407 cells were dissociated by treatment with trypsin and overlaid, in 55mm petrie dishes, with molten LBA (0.7% agar) supplemented with antibiotics as indicated. The donors were $SL1344^{R}$ (RP4) and the recipients were $SL1344^{N}$.

✓ = cell-associated colonies observed; x = cell-associated colonies not observed.

[b]Not determined.

A small number of Nx- and Kn-resistant cell-associated bacterial colonies were observed when cultured cells were infected sequentially with recipients then donors in intracellular conjugation assays (as in Chapter 3) (Table 2). Three representative colonies were dissected out of the LBA medium with a sterile needle, diluted in PBS and plated on LBA supplemented with either Rf or Nx+Kn for enumeration of donor and transconjugant bacteria. Donor bacteria were not recovered in significant numbers, but the cell-associated colonies were found to contain, on average, 2×10^7 transconjugants.

In order to determine the proportion of INT-407 cells containing donors, recipients and transconjugants in the intracellular conjugation experiments presented in Chapter 3, these experiments were repeated with the inclusion of replicates that were unlysed and immobilized in LBA as described above (Table 3). The titre of cells infected with either donor or recipient bacteria was determined by overlaying serial dilutions of infected cells with LBA supplemented with the appropriate antibiotics. Thus, the proportion of cells infected with donors, recipients or transconjugants could be estimated by dividing the number of infected cells (as a cell-associated colony titre) by the total number of cells (determined by haemocytometer counts). By calculation, an average of 58% of INT-407 cells were infected with recipient bacteria at $\pm$ 21 bacteria per cell and 7% of INT-407 cells were infected with donor bacteria at $\pm$ 34 bacteria per cell. Assuming that donors were not more likely to invade cells pre-infected with recipients than they were uninfected cells (although the reverse may be true), the proportion of cells infected with both donors and recipients can be estimated at 0-4%. The calculated proportion of co-infected cells containing transconjugants is thus $\leq$0.25%.

TABLE 3. Transconjugant formation within immobilized cells[a]

				% INT407 infection [e]		
Strain	Expt	Total TC[b] titre[c]	TC$^+$ cells[d]	Recipients	Donors	TCs
SL1344	1	87 ± 13 (1×10^{-4})[f]	45.5 ± 4.5	50 (15)[g]	3.25 (47)[g]	0.01 (1.9)[g]
	2	329 ± 40 (1×10^{-4})	149 ± 14	97 (16)	15 (15)	0.01 (2.2)
	3	237 ± 24 (1×10^{-4})	110.5 ± 2.1	28 (31)	3.8 (40)	0.007 (2.1)
	Avg.	218 ± 61	102 ± 26	58 (21)	7 (34)	0.009 (2.1)
SR11	1	251 ± 56 (1×10^{-3})	67.5 ± 3.7	33 (33)	2 (12)	0.008 (3.7)
	2	451 ± 71 (9×10^{-4})	113.5 ± 47	100 (8)	10 (12)	0.03 (4.0)
	3	130 ± 10 (6×10^{-4})	42.5 ± 6.5	28 (30)	1.8 (23)	0.009 (3.1)
	Avg.	277 ± 81	74.5 ± 18.0	54 (24)	4.6 (16)	0.01 (3.6)

[a] Donor *S. typhimurium* were either SL1344^R (RP4) or SR11^R (RP4) as indicated. Recipient *S. typhimurium* were either SL1344^N or SR11^N. Each experiment was performed in triplicate.

[b]Transconjugant.

[c]Cells were lysed and the lysates plated on LB agar plates supplemented with Nx and Kn to determine the total number of transconjugants per well.

[d]Cells were detached by treatment with trypsin and mixed with molten LB agar supplemented with Nx and Kn to determine the total number of cells (as cell-associated colonies per well) containing transconjugants (TC$^+$ cells).

[e]The number of INT-407 cells per well was determined by haemocytometer counts. The percentage infection was calculated by dividing ‘cell associated colonies per well’ by the ‘total number of cells per well’.

[f]The numbers in parentheses are transconjugant frequencies (TCs per limiting parent).

[g]The numbers in parentheses are the average number of bacteria per infected cell.

The experiment was repeated substituting SR11 for SL1344. This was because SR11 was found to transmit RP4 to *S. typhimurium* recipients at a 10-fold greater frequency than SL1344, both on LB agar plates and within INT-407 cells. The high frequency appears to be due to poor RP4 transfer from SL1344 rather than poor transmission of RP4 to the SL1344 background (data not shown). If “epidemic spread” of RP4 to all accessible recipients within a small number of permissive co-infected cells were the predominant source of transconjugants (‘permissive cells’ being those where physical contact between donors and recipients potentiated conjugation) then it might be predicted that the ratio of total transconjugant colonies to cell-associated transconjugant colonies be 10-fold higher for SR11 parents than for SL1344 parents. The average number of transconjugants per cell was ±2.1 for SL1344 and ±3.6 for SR11, suggesting that rather than a failure of SL1344 exconjugants to disseminate RP4 within a small number of permissive cells, conjugation failed to occur in a proportion of a larger population of permissive cells.

Is the ‘unlysed cell immobilization assay’ a reliable indicator of the intracellular distribution of transconjugants? The reliability of the immobilization assay as an indicator of percentage infection, percentage co-infection and transconjugant distribution is questionable. The assay is based upon two major assumptions: firstly, that every infected cell results in a cell-associated colony when overlaid with medium permissive for growth, and secondly that cell-associated transconjugant colonies initiate from transconjugants preformed at the time of cell immobilization and not events initiated *after* plating of the infected cells. Although cell-associated transconjugant colonies do not result when donor- and recipient-infected cells are mixed at the time of immobilization (Table 2), it is possible that slow diffusion of antibiotics into co-infected cells allows nascent conjugation events to continue to occur for a length of time following immobilization of the cells.

The first assumption was tested by fluorescence microscopy. Donor- and recipient-infected cells grown on glass cover slips were fixed, stained with DAPI and the

proportion of infected cells counted on an epifluorescence microscope. 78% of cells were estimated to be infected with recipient *S. typhimurium* ($SR11^N$) with a highly variable number of bacteria per cell: 12% of infected cells contained moderately large clusters of 20-30 bacteria, 2% of cells contained an incalculably large number of bacteria (these will be discussed later) and the remaining 86% of infected cells contained small clusters (< 20 bacteria) or single bacteria. Likewise, 41% of cells were infected with donor *S. typhimurium* [$SR11^R$ (RP4)] at an average of 3.4 bacteria per cell. In contrast, an immobilization assay performed in parallel estimated only 28% of cells to be infected with recipients at ± 30 bacteria per cell and only 1.8% of cells infected with donors at ± 23 bacteria per cell (Table 3, SR11 expt. 3). Thus, it appears that the immobilization assay underestimates the true number of infected cells.

The second assumption was tested by comparing the number of cell-associated transconjugant colonies between infected cells immobilized three hours and those immobilized seven hours following introduction of donors to the cell culture (Table 4). While the total number of transconjugant bacteria was 8-fold less at the earlier time point, the corresponding number of cell-associated transconjugant colonies was down only 1.6-fold. Further, the ratio of total transconjugants to cell-associated transconjugant colonies was significantly less than one at 3 hours (compared with 2.1 at 7 hours). Since a TC^+ (transconjugant-containing) cell cannot contain less than one transconjugant, the cell immobilization assay overestimates the number of TC^+ cells, presumably by failing to prevent nascent plasmid transmission events from occurring after plating of the infected cells. Thus, an alternative assay for determining the intracellular distribution of transconjugants was sought.

TABLE 4. Comparison of transconjugant formation within cells immobilized three and seven hours following the internalization of donors[a]

		3 hours[b]		7 hours[b]	
Strain	Expt	Total TC[c] titre[d]	TC$^+$ cells[e]	Total TC[c] titre[d]	TC$^+$ cells[e]
SL1344	1	6.7 ± 0.6 (2×10^{-5})[f]	24.5 ± 1.5	87 ± 13 (1×10^{-4})[f]	45.5 ± 4.5
	2	54.6 ± 2.9 (3×10^{-5})	61.0 ± 5.5	329 ± 40 (1×10^{-4})	149.0 ± 14.0
	3	19.0 ± 5.0 (2×10^{-5})	100.5 ± 10.0	237 ± 24 (1×10^{-4})	110.5 ± 2.1
	Avg.	27 ± 12	62 ± 19	218 ± 61	102 ± 26
SR11	1	53 ± 8 (3×10^{-4})	13.0 ± 0.5	251 ± 56 (1×10^{-3})	67.5 ± 3.7
	2	27 ± 10 (1×10^{-4})	34.5 ± 6.0	451 ± 71 (9×10^{-4})	113.5 ± 47.0
	3	24 ± 1 (1×10^{-4})	23.0 ± 2.5	130 ± 10 (6×10^{-4})	42.5 ± 6.5
	Avg.	35 ± 8	24 ± 5	277 ± 81	75 ± 18

[a]Donor *S. typhimurium* were either SL1344^R (RP4) or SR11^R (RP4) as indicated. Recipient *S. typhimurium* were either SL1344^N or SR11^N. Each experiment was performed in triplicate.

[b]Cell lysates and unlysed cells were plated to select for transconjugants immediately after Gm-killing of extracellular donors (3 hours after introduction of donors to the cell culture) and again following the usual experimental time course (7 hours after introduction of donors to the cell culture).

[c]Transconjugants.

[d]Cells were lysed and the lysates plated on LB agar plates supplemented with Nx and Kn to determine the total number of transconjugants per well.

[e]Cells were detached by treatment with trypsin and mixed with molten LB agar supplemented with Nx and Kn to determine the total number of cells (as cell-associated colonies per well) containing transconjugants.

[f]The numbers in parentheses are transconjugant frequencies (TCs per limiting parent).

Design of a genetic, fluorescence-based, assay for determining the intracellular distribution of transconjugants. The application of fluorescence-activated cell sorting (FACS) to the detection of GFP induction amongst bacteria located within cultured cells has proved a highly successful strategy for the discovery of intracellularly-induced genes (Valdivia *et al.*, 1996; Valdivia and Falkow, 1996; Valdivia and Falkow, 1997). Large numbers of bacteria carrying random promoter-trap fusions to GFP can be easily screened for rare individuals displaying intracellular fluorescence. It was anticipated that FACS might therefore be successfully applied to the detection of intracellular transconjugants. To this end, we designed an assay to detect gene transfer by induction of GFP expression specifically within recipient *S. typhimurium* who receive a reporter gene from donor *S. typhimurium*. Selectivity of reporter induction was achieved by repression of reporter expression within the donor bacterium. A fusion of the *S. typhimurium* SPI-2 type III secretion gene *ssaH* promoter to *gfp* was chosen as a suitable reporter system (Valdivia and Falkow, 1997). *ssaH* is induced within intracellular *S. typhimurium* and induction is mediated by the two component regulatory system encoded by *ssrA* and *ssrB*. *S. typhimurium* encoding functional SsrA and SsrB and carrying *ssaH::gfp* fluoresce green upon entry into cultured cells. Since the SPI-2 TTS system is essential only for bacterial survival inside murine macrophages (Brumell *et al.*, 2001a), SsrA and SsrB are not expected to be necessary for invasion of epithelial cells. Thus, *ssaH::gfp* expression within donor *S. typhimurium* likely can be repressed by mutation of *ssrA* or *ssrB* without compromising the ability of the bacterium as an intracellular plasmid donor.

A small deletion marked by insertion of a Cm^R marker was created in the *ssrB* gene of the strain SR11. $SR11^R_{ssrB::Cm}$ invaded INT-407 cells with equal proficiency to $SR11^R$ (data not shown) but did not express a detectable level of SsaH::GFP (Fig. 3). *ssaH::gfp* is encoded on the plasmid pFM10.1::Tc. pFM10.1::Tc encodes IncQ *mob* functions and *oriT* and is thus mobilizable in *trans* by the RP4 derivative pRK2526. pRK2526 mobilizes pFM10.1::Tc to recipient bacteria at a frequency ranging from 2- to 5-fold below that of its own transmission (data not shown). It was

predicted that conjugative transfer of pFM10.1::Tc to wild-type *S. typhimurium*, within cultured cells, would result in *ssaH::gfp* induction and the observation of green fluorescent transconjugants. Infected INT-407 cells could be analyzed and sorted by Fluorescence-Activated Cell Sorting (FACS), which detects and enumerates cells on the basis of subcellular fluorescence.

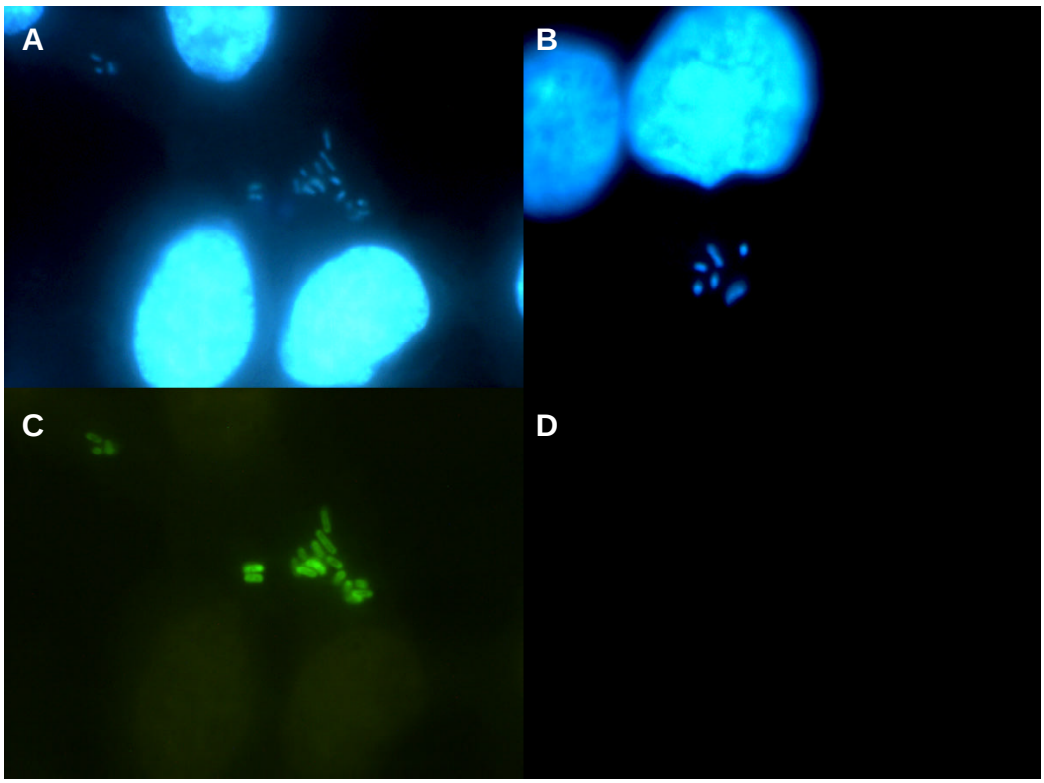


FIG. 3. *S. typhimurium ssrB* mutants do not express *ssaH::gfp*. INT-407 cells were infected with either $SR11^{R}$ (panels A and C) or $SR11^{R}_{ssrB::Cm}$ (panels B and D) carrying pFM10.1::Tc encoding GFP under control of the SsrB-regulated promoter *ssaH*. Upper panels (A and B) show DAPI fluorescence, lower panels (C and D) show GFP fluorescence.

SR11$^{R}_{ssrB::Cm}$ donors carrying pFM10.1::Tc and pRK2526 were mated intracellularly with SR11^{N} recipients. In contrast to previous intracellular conjugation experiments (Chapter 3), only a small number of pRK2526 transconjugants were detected and, in most experiments, pFM10.1::Tc recombinants were not detected at all (Table 5). This was attributed to low invasiveness of the donor strain. *S. typhimurium* carrying pFM10.1::Tc (and other pUC-based vectors) generally invaded cells at a 10-fold lower efficiency than plasmid-free bacteria (data not shown). The detrimental effect of pUC vectors on invasion appeared to be exacerbated by carriage of the conjugative plasmid pRK2526. Similar results were obtained when pRK2526 was substituted for the IncPβ plasmid R751 (data not shown). SR11$^{R}_{ssrB::Cm}$ (pRK2526, pFM10.1::Tc) cultures grew significantly slower than SR11^{R} (pFM10.1::Tc) cultures and although the slow-growing donor cultures were incubated until they reached the same density as wild-type bacteria, the bacteria invaded poorly. The threshold of intracellular SR11$^{R}_{ssrB::Cm}$ (pRK2526, pFM10.1::Tc) required for detection of intracellular transconjugants was never achieved (Table 5).

Since carriage of pRK2526 alone did not compromise *S. typhimurium* invasion, it was predicted that both the invasion proficiency and transmission frequency of *ssaH::gfp* could be improved if the *ssaH::gfp* construct was incorporated into pRK2526. Two directed strategies for integration of *ssaH::gfp* into the *lacZ* gene on pRK2526 (e.g. Datsenko and Wanner, 2000), as well as random mutagenesis using miniTn*10*::*ssaH::gfp* (Heinemann *et al.*, 1996), were carried out. Although putative pRK2526::*ssaH::gfp* recombinant plasmids were isolated, these failed to confer green fluorescence upon host *S. typhimurium*, even when resident within INT-407 cells for ten hours (data not shown). Similarly, efforts to clone functional *ssaH::gfp* onto the low copy number mobilizable RSF1010-derivative pMMB207αB (Segal and Shuman, 1997) [using standard cloning procedures (Sambrook *et al.*, 1989)] were also unsuccessful. Lack of expression may have been due to either structural rearrangements or mutation within the cloned *ssaH::gfp* construct. Alternatively, the low copy number of pRK2526::*ssaH::gfp* may have limited the

accumulation of a detectable concentration of Ssa::GFP. The reasons for the failure of these cloning procedures were not determined.

TABLE 5. Inefficient donor internalization prevented the formation of intracellular *ssaH::gfp* transconjugants

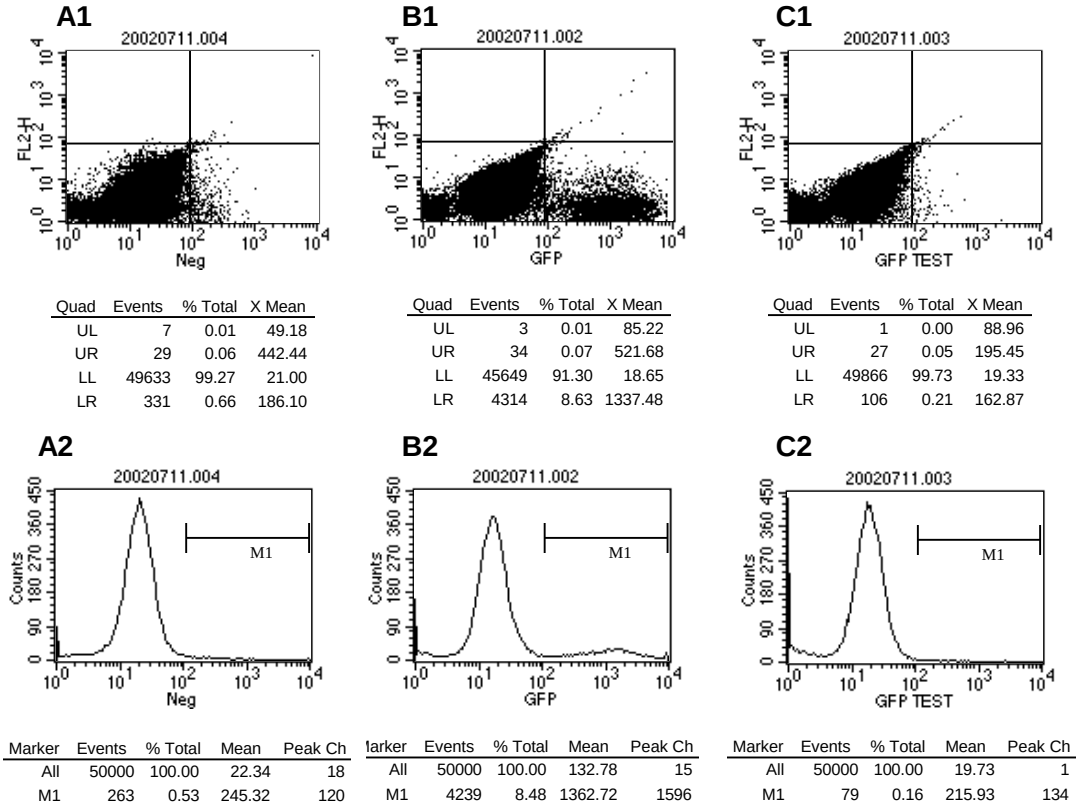
	Intracellular bacteria[a]		Transmission frequency[b]	
Expt	Donors	Recipients	pRK2526	pFM10.1::Tc
1	$3 \times 10^3 \pm 6 \times 10^2$	$2 \times 10^6 \pm 4 \times 10^5$	1×10^{-3} (12)[c]	$\leq 7 \times 10^{-4}$ (0)
2	$1 \times 10^3 \pm 2 \times 10^2$	$2 \times 10^6 \pm 3 \times 10^5$	2×10^{-3} (4)	$\leq 2 \times 10^{-3}$ (0)
3	$5 \times 10^4 \pm 1 \times 10^4$	$5 \times 10^6 \pm 9 \times 10^5$	1×10^{-3} (192)	$\leq 4 \times 10^{-5}$ (0)
4	$2 \times 10^5 \pm 4 \times 10^4$	$5 \times 10^6 \pm 8 \times 10^5$	3×10^{-4} (80)	3×10^{-5} (9)
5	$1 \times 10^4 \pm 5 \times 10^3$	$2 \times 10^6 \pm 6 \times 10^5$	9×10^{-4} (9)	3×10^{-4} (4)
6	$2 \times 10^4 \pm 6 \times 10^3$	$7 \times 10^6 \pm 2 \times 10^6$	2×10^{-4} (7)	$\leq 1 \times 10^{-4}$ (0)

[a]Cfu/well. Donors were $SR11^R_{ssrB::Cm}$ (pRK2526, pFM10.1::Tc) and recipients were $SR11^N$. Each experiment was performed in triplicate.

[b]Transconjugants per limiting parent.

[c]Numbers in parentheses are the total number of transconjugant colonies.

In spite of the low pFM10.1::Tc transconjugant plate titres, FACS analysis of infected INT-407 cells from intracellular mating experiments was still performed, predominantly as an exercise in exploring the suitability of such an assay for the detection of rare gene transfer events within cultured cells. A representative FACS experiment is depicted in Figure 4. A sample size of 5×10^4 INT-407 cells from each population (positive control, negative control, test population) was analyzed for GFP fluorescence. A population of cells infected with $SR11^R$ (pFM10.1::Tc, pRK2526) control bacteria were positive for green fluorescence (Fig. 4). However, no green fluorescence above that detected within the sample infected with $SR11^R_{ssrB::Cm}$ (pFM10.1::Tc, pRK2526) alone was detected within the co-infected (intracellular conjugation) sample.



Quad	Events	% Total	X Mean
UL	7	0.01	49.18
UR	29	0.06	442.44
LL	49633	99.27	21.00
LR	331	0.66	186.10

Quad	Events	% Total	X Mean
UL	3	0.01	85.22
UR	34	0.07	521.68
LL	45649	91.30	18.65
LR	4314	8.63	1337.48

Quad	Events	% Total	X Mean
UL	1	0.00	88.96
UR	27	0.05	195.45
LL	49866	99.73	19.33
LR	106	0.21	162.87

Marker	Events	% Total	Mean	Peak Ch
All	50000	100.00	22.34	18
M1	263	0.53	245.32	120

Marker	Events	% Total	Mean	Peak Ch
All	50000	100.00	132.78	15
M1	4239	8.48	1362.72	1596

Marker	Events	% Total	Mean	Peak Ch
All	50000	100.00	19.73	1
M1	79	0.16	215.93	134

FIG. 4. Fluorescence-activated cell sorting (FACS) of INT-407 cells infected with *S. typhimurium* carrying plasmid-borne fusions of *gfp* to *ssaH*. INT-407 cells infected with $SR11^{R}_{ssrB::Cm}$ (pRK2526, pFM10.1::Tc)(A, negative control), $SR11^{R}$ (pRK2526, pFM10.1::Tc) (B, positive control) or co-infected with $SR11^{N}$ and $SR11^{R}_{ssrB::Cm}$ (pRK2526, pFM10.1::Tc) in an intracellular conjugation assay (C) were analyzed by flow cytometry. Panels A1, B1 and C1 show the scatter plot of the analyzed cells, with the GFP fluorescence intensity plotted on the x-axis and side scatter plotted on the y-axis. The data is represented as histograms in the lower panels (A2-C2). Events that graph within the region labeled 'M1' were designated 'fluorescent'.

Determining the intracellular location of transconjugants. As discussed previously, two models for how donor and recipient bacteria form the physical contacts necessary for conjugative plasmid transfer were proposed: the ‘vacuole coalescence model’ and the ‘vacuole escape model’ (Fig. 1). These were tested by engineering *S. typhimurium* strains that either actively lyse the SCV or are released into the cytoplasm upon failing to maintain the integrity of the vacuolar membrane.

Mutation in *sifA* does not affect the frequency of intracellular conjugation. Two predictions can be made regarding the effect of cytoplasmic release of *sifA* mutants on the potential for intracellular conjugation. Firstly, that the effect of *sifA* on conjugation potential should be greatest when *sifA* mutations are carried by the recipient and secondly, that the effect of *sifA* on conjugation potential should increase with the length of time that recipient-infected cells are incubated prior to the introduction of donors. These predictions are based on the known time-dependence of SCV degeneration (Beuzón *et al.*, 2000; Brumell *et al.*, 2002) and the assumption that a *sifA* mutant will be “rescued” by fusion of its containing vacuole with that of a wild-type bacterium. Recipients, being internalized by cultured cells some hours prior to donors in our assay, should increase their cytoplasmic predominance with time and be unaffected by the introduction of wild-type bacteria after they relocate to the cytoplasm. Thus, in test of the vacuolar fusion model, wild-type *S. typhimurium* donors were mated intracellularly with *S. typhimurium sifA* recipients with varied length of incubation prior to donor internalization (Table 6). The experiments were carried out within MDCK cells since these proved more resilient to the detrimental effects of intracellular bacterial replication over the longer incubation times of these experiments, an observation noted by others (Stein *et al.*, 1996).

In the experiments carried out with SL1344 parent strains, there was a small but consistent decrease in the frequency of plasmid transmission to *sifA* recipients (Table 6). Curiously, the effect of the *sifA* mutation appeared to be enhanced in the shorter duration experiments, contrary to prediction. This effect was not observed

using SR11 parents carrying a transposon insertion in *sifA* (Table 6). Interestingly, the SL1344$_{\Delta sifA}$ strain appeared to be compromised not as a recipient but as a *donor* of RP4 (Table 7). The frequency of RP4 transfer from Δ*sifA* SL1344 donors was decreased by a factor of 5. Thus, the reduction in intracellular RP4 transmission to Δ*sifA* recipients is likely due to less extensive dissemination of the plasmid by Δ*sifA* exconjugants.

TABLE 6. Intracellular plasmid transmission to *sifA*$^-$ recipients

Expt	Transmission frequency[a]			
	SL1344^R (RP4) × SL1344^N	SL1344^R (RP4) × SL1344$^N_{\Delta sifA}$	SR11^R (RP4) × SR11^N	SR11^R (RP4) × SR11$^N_{sifA::Kn}$
1 (3 hrs)[b]	$7 \times 10^{-4} \pm 5 \times 10^{-5}$ (1866)[c]	$1 \times 10^{-4} \pm 3 \times 10^{-5}$ (423)	ND[d]	ND
2 (3hrs)	$2 \times 10^{-3} \pm 3 \times 10^{-4}$ (4961)	$4 \times 10^{-4} \pm 8 \times 10^{-5}$ (1301)	ND	ND
3 (7hrs)	$6 \times 10^{-4} \pm 5 \times 10^{-5}$ (3425)	$2 \times 10^{-4} \pm 8 \times 10^{-5}$ (1218)	$2 \times 10^{-3} \pm 3 \times 10^{-4}$ (539)	$3 \times 10^{-3} \pm 6 \times 10^{-4}$ (331)
4 (7hrs)	$2 \times 10^{-4} \pm 5 \times 10^{-5}$ (3659)	$7 \times 10^{-5} \pm 2 \times 10^{-5}$ (1360)	$2 \times 10^{-3} \pm 8 \times 10^{-4}$ (701)	$1 \times 10^{-3} \pm 3 \times 10^{-4}$ (674)
5 (10hrs)	ND	ND	$3 \times 10^{-3} \pm 1 \times 10^{-3}$ (531)	$1 \times 10^{-3} \pm 4 \times 10^{-4}$ (51)[e]
6 (12hrs)	$2 \times 10^{-3} \pm 5 \times 10^{-4}$ (94)	$5 \times 10^{-4} \pm 6 \times 10^{-5}$ (60)	No result	No result
7 (12hrs)	No result	No result	ND	ND

[a] Number of transconjugants per limiting parent. The frequencies are averages ± standard deviations of experiments performed in triplicate. The frequency of plasmid transmission between bacteria on the enumeration plates ranged from 10-1000-fold below the intracellular plasmid transmission frequencies reported here (data not shown).

[b]Numbers in parentheses are the maximum number of hours that recipient *S. typhimurium* resided within the cultured cells prior to the introduction of donor bacteria (the minimum number of hours thus being two hours less since recipients were allowed two hours to invade prior to washing and incubation in gentamicin).

[c]Numbers in parentheses are the total number of transconjugant colonies observed.

[d]Experiment not done.

[e]In this experiment, the significantly smaller number of transconjugant colonies was a consequence of 10-fold lower bacterial invasion in the *sifA* mating experiment relative to the control experiment.

TABLE 7. Plasmid transmission frequencies to and from *sifA*⁻ *S. typhimurium* mixed on LB agar plates[a]

Donor		Recipient	Transmission frequency [b]
SL1344^{R} (RP4)	×	SL1344^{N}	0.1 ± 0.05[c]
SL1344^{R} (RP4)	×	SL1344^{N} $_{\Delta sifA}$	0.1 ± 0.05[c]
SL1344^{R} $_{\Delta sifA}$ (RP4)	×	SL1344^{N}	0.05 ± 0.004[c]
SL1344^{R} $_{\Delta sifA}$ (RP4)	×	SL1344^{N} $_{\Delta sifA}$	0.03 ± 0.005[c]
SL1344^{R} $_{sifA::km}$ (RP4)	×	SL1344^{N}	0.25 ± 0.09[d]
SR11^{R} (RP4)	×	SR11^{N}	1.8 ± 1.0[d]
SR11^{R} (RP4)	×	SR11^{N} $_{sifA::km}$	1.7 ± 0.6[d]
SR11^{R} $_{sifA::km}$ (RP4)	×	SR11^{N}	1.7 ± 0.6[d]

[a] Bacteria were mixed for one hour on LB agar plates at 37°C.

[b] Number of transconjugants per limiting parent. The frequencies are averages ± standard deviations based on one[d] or two[c] independent experiments performed in triplicate.

Do S. typhimurium sifA mutants escape the vacuole? Having established that the *sifA* mutation did not affect the ability of recipients to partake in intracellular conjugation, the kinetics of *sifA* mutant vacuolar escape was examined. Brumell *et al.* reported that a significant proportion of *sifA*⁻ *S. typhimurium* were located in the cytoplasm 10 hours following entry into cultured epithelial cells (Brumell *et al.*, 2002). *sifA* mutants replicated extensively within the cytoplasm and numerous cells whose cytoplasmic space was completely filled with bacteria were observed (Brumell *et al.*, 2002). Cytoplasmic bacteria were typically elongated, motile and displayed down-regulation of a reporter fusion of the intracellularly-induced protein PhoP to an unstable variant of GFP (having a half-life of 30 minutes) (Brumell *et al.*, 2002). *phoP* is specifically induced within the vacuole in response to a low concentration of Mg^{+2} (Lee *et al.*, 2000). Thus, movement of bacteria into the cytoplasm is followed by a rapid reduction in the intensity of GFP fluorescence (Brumell *et al.*, 2002). Hereon, 'reduced expression of *phoP::gfp*' refers to a

reduction in GFP fluorescence intensity as observed by epifluorescence microscopy. Transcription and translation of *phoP::gfp* was not measured directly in these experiments.

MDCK cells were infected with SR11^{R} or SR11$^{R}_{sifA::Km}$ carrying pMLZ205, which encodes the *phoP::gfp* reporter construct. Both the growth of intracellular bacteria (Fig. 5) and expression of PhoP::GFP (Figs. 6, 7 and 8) was determined over time.

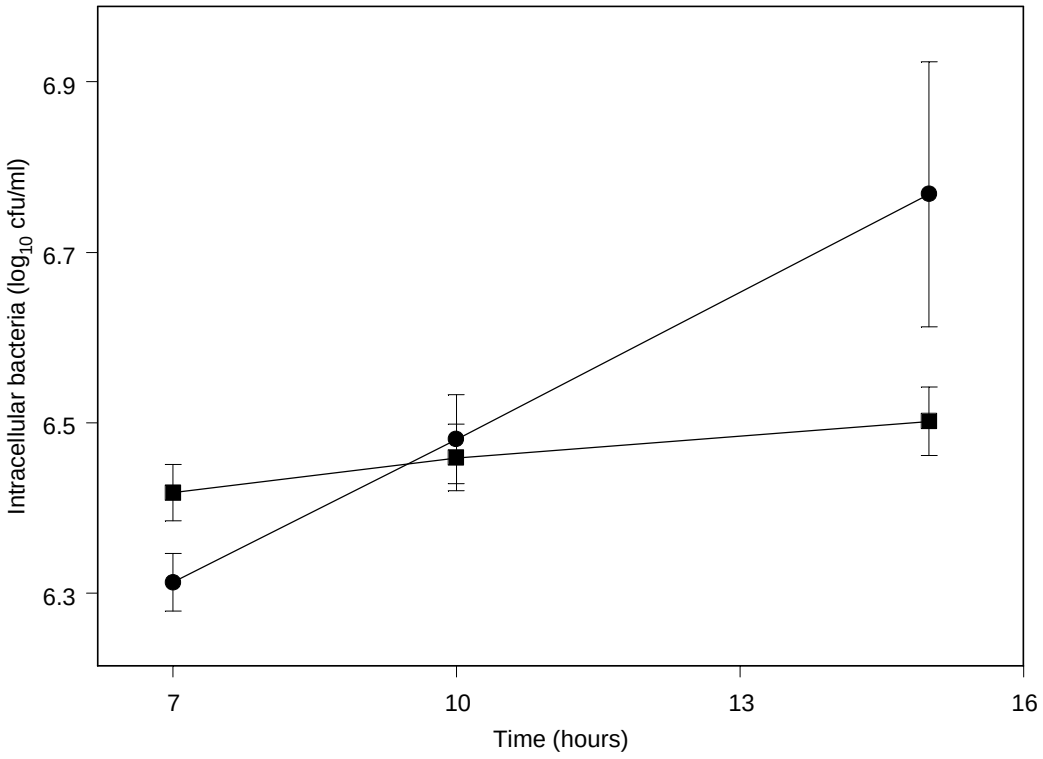


FIG.5. Intracellular growth of *sifA*$^{-}$ *S. typhimurium*. INT-407 cells were infected with either SR11^{N} (pMLZ205) (¦) or SR11$^{N}_{sifA::km}$ (pMLZ205) (?) and the numbers of intracellular bacteria determined after 7, 10 and 15 hours by plating serial dilutions of cell lysates on LB agar plates. Each time point represents an average of three replicates.

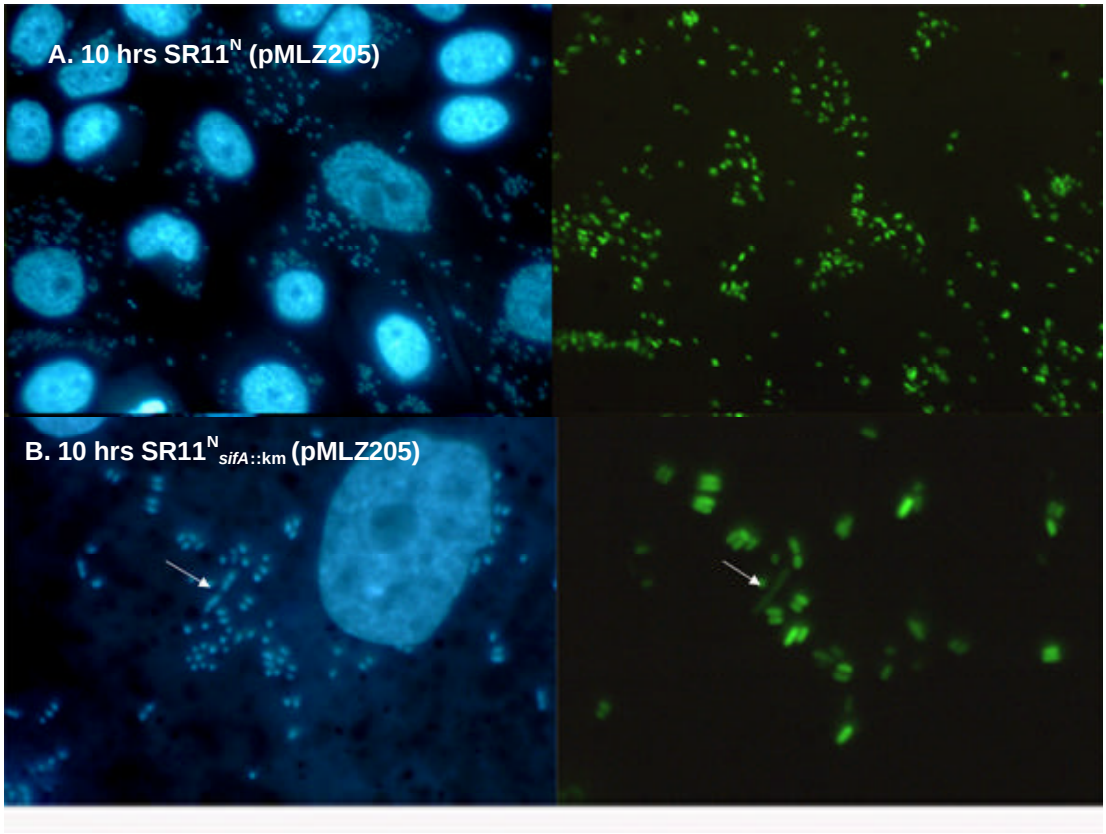


FIG.6. *sifA S typhimurium* begin to display cytoplasmic characteristics ten hours following internalization. While most *sifA*$^{-}$ bacteria were indistinguishable from wild-type bacteria (A) after residing within MDCK cells for ten hours (data not shown), there was a small incidence of *phoP::gfp* down-regulation and elongation (B, indicated by arrows) amongst a small proportion of *sifA*$^{-}$ bacteria.

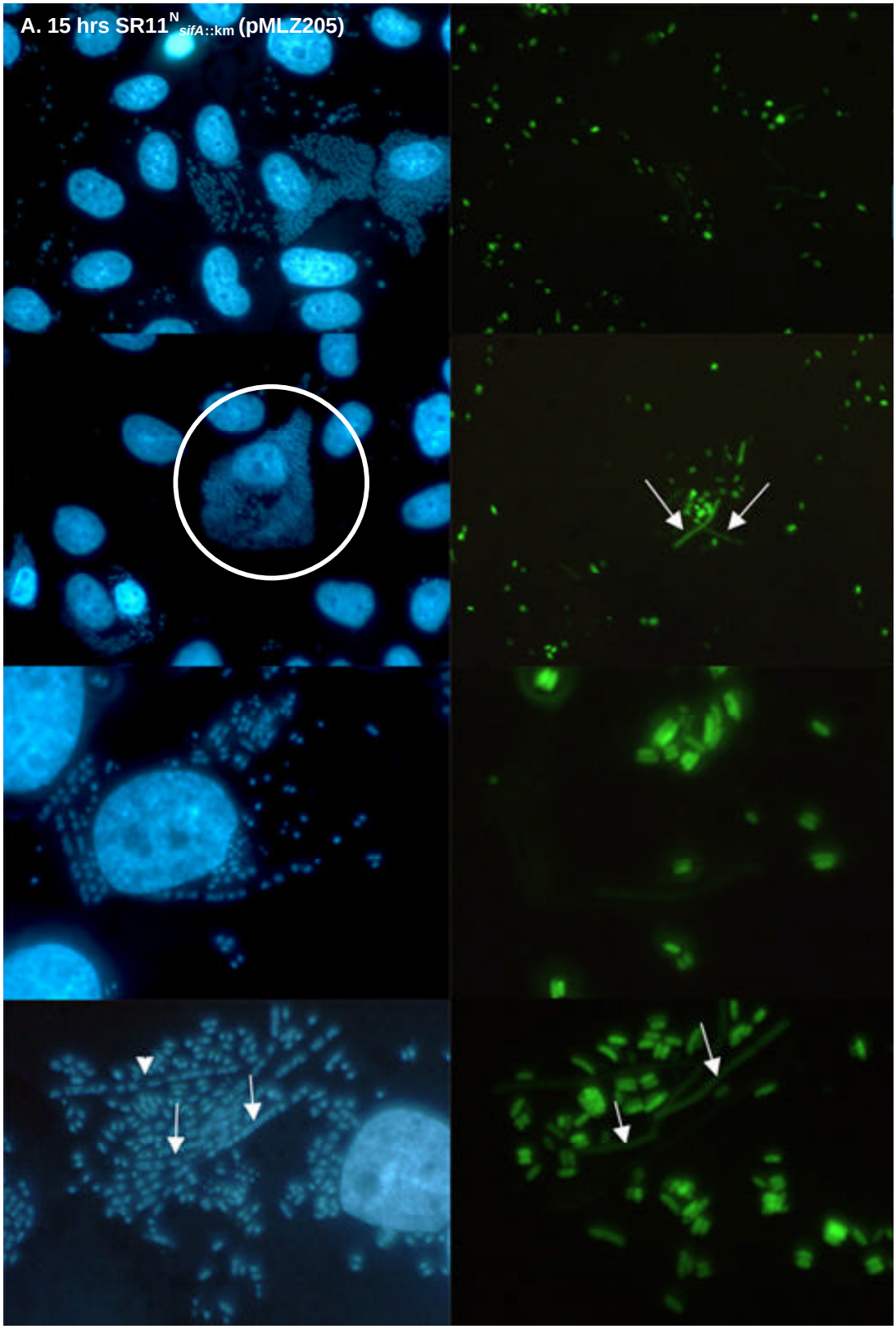


FIG.7A. (see the following page for the legend).

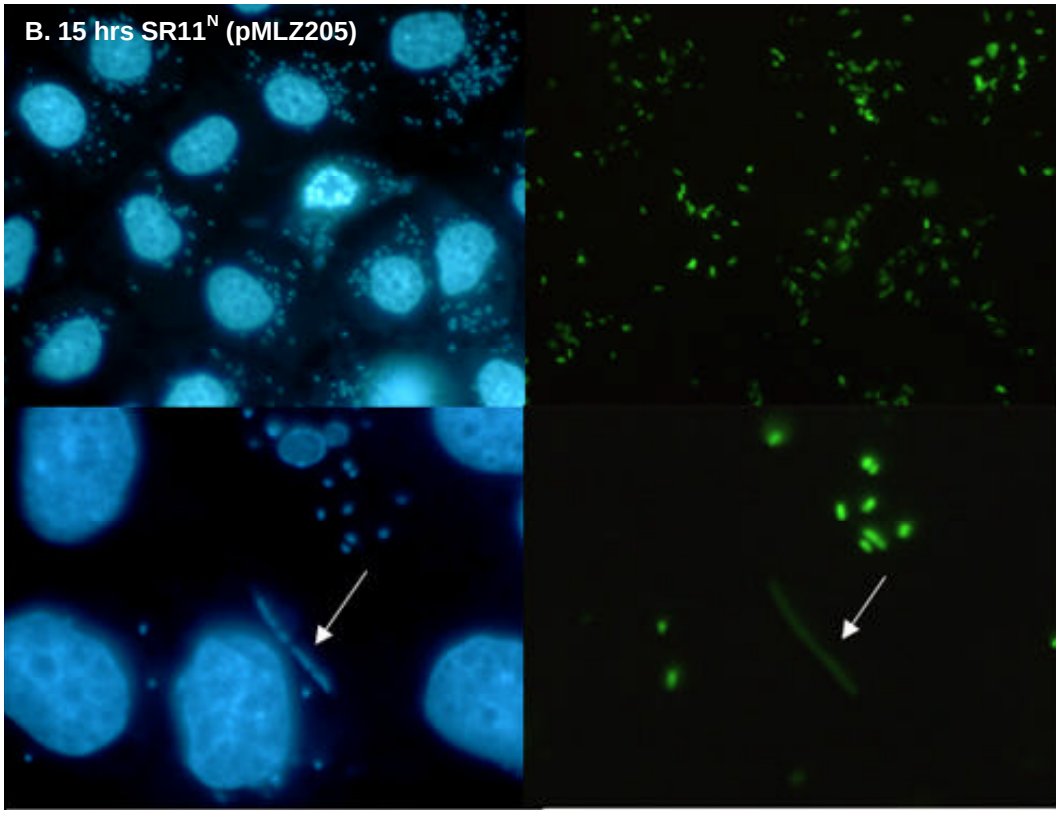


FIG.7B. *sifA*⁻ *S typhimurium* display significant intracellular growth and cytoplasmic characteristics fifteen hours following internalization. After residing within MDCK cells for fifteen hours, a subpopulation of *sifA*⁻ bacteria ceased to fluoresce, presumably due to down-regulation of *phoP::gfp* (A). GFP down-regulation was largely co-incident with the appearance of elongated bacteria (A, indicated by arrows) and extensive replication of bacteria to occupy the entire cytoplasmic space (A, ringed), a phenomenon not apparent at the earlier time points (7 and 10 hours). In contrast, wild-type bacteria appeared largely unchanged from the earlier time points (B) although a low incidence of bacterial elongation and decreased fluorescence was evident (B, indicated by arrows).

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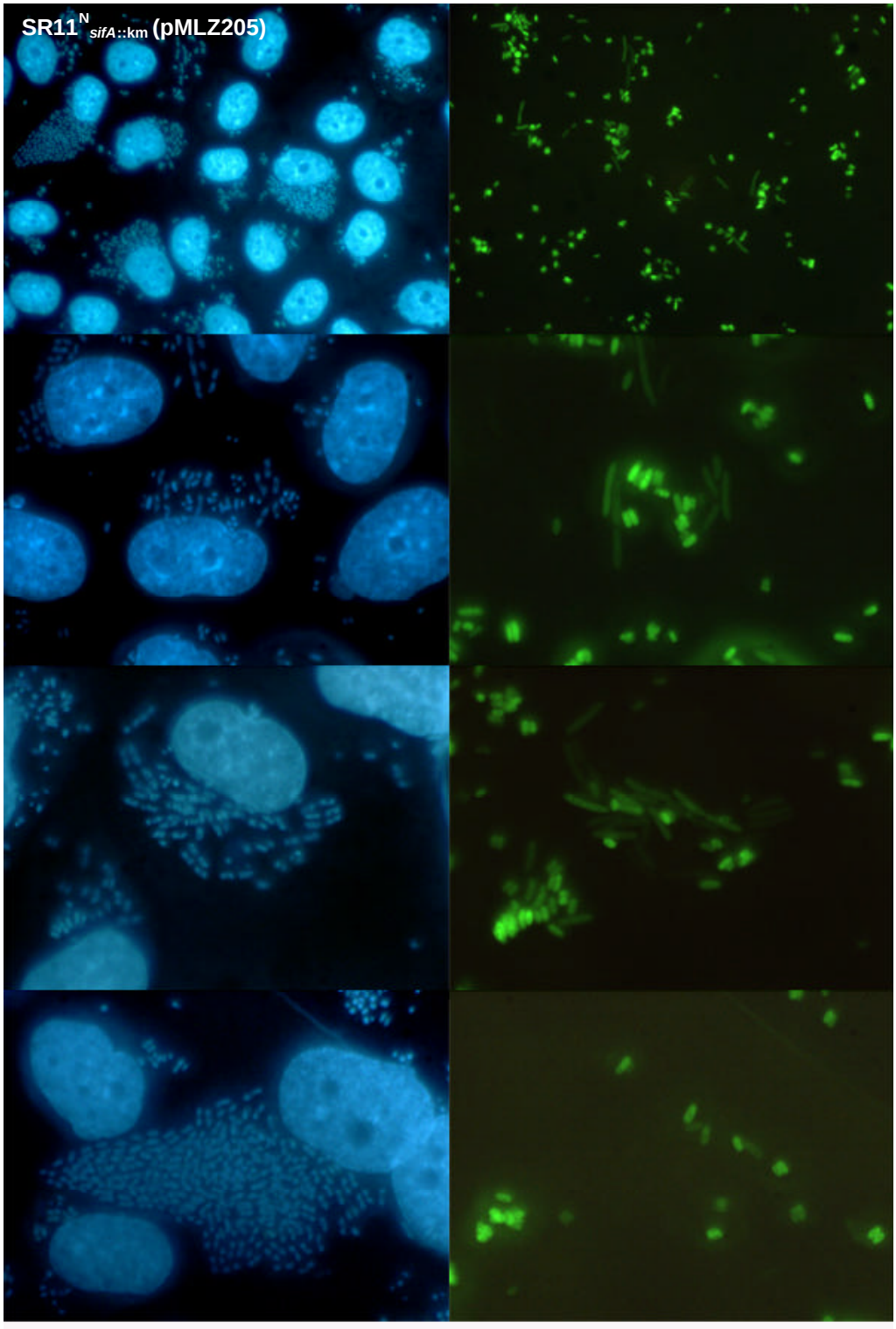

FIG.8. The length of time before observation of *sifA*⁻ characteristics varied between experiments. In a second experiment, *sifA*⁻ cytoplasmic characteristics were readily apparent 10 hours following entry of bacteria into MDCK cells.

As reported by others (Brumell *et al.*, 2002), a proportion of intracellular *sifA* mutants displayed reduced PhoP::GFP fluorescence, elongated morphology and significant replication within some infected cells (Figs. 7 and 8). The *sifA*$^-$ characteristics were first apparent 10 hours following invasion (Fig. 6), being more pronounced in some experiments than others at this time (Fig. 8), and were readily apparent 15 hours following invasion (Fig. 7).

No difference in the frequency of plasmid transmission to wild-type and *sifA*$^-$ recipients was observed when recipients were incubated within MDCK cells for up to 10 hours prior to the first internalization of donors (Table 6). This corresponded with the observation of bright GFP fluorescence and wild-type morphologies for the majority of *sifA*$^-$ bacteria over this time period (Fig. 6). The lack of *sifA*$^-$ effect on the plasmid transmission frequency could therefore be sufficiently explained by inefficient vacuolar escape of the *sifA*$^-$ recipients.

Is the extent of vacuolar escape sufficient to detect a change in plasmid transmission frequency to sifA$^-$ *recipients?* At least a 10-fold decrease in the number of vacuolar bacteria is likely to be necessary to confidently detect a change in plasmid transmission frequency. Although a greater proportion of *sifA*$^-$ recipients displayed cytoplasmic characteristics 15 hours after entry into MDCK cells, attempts to extend the incubation time to 15 hours before internalization of donors were unsuccessful. The detrimental effect of extensive *sifA*$^-$ *S. typhimurium* replication within MDCK cells after this length of co-incubation prevented donor invasion (indicated as ‘expt 6’, Table 6). After 15 hours of co-incubation, cells infected with *sifA* mutants appeared bloated with large numbers of motile bacteria visible within a large number of cells.

It was hypothesized that release of intracellular *S. typhimurium* from the SCV by active lysis of the vacuole membrane may result in a more rapid and extensive cytoplasmic relocation of bacteria, thus being more amenable to test of the vacuolar coalescence model.

***ipaC* complements *S. typhimurium sipC* mutants insufficiently for test of the vacuole fusion model.** IpaC secretion mediates both bacterial internalization and rapid vacuolar lysis in *S. flexneri* (Osiecki *et al.*, 2001). Thus, cytoplasmic release of *S. typhimurium* whose invasion is mediated by IpaC should too be rapid and likely more extensive than that mediated by mutation of *sifA*.

S. typhimurium sipC deletion mutants were created and tested for *sipC* and *ipaC* complementation in *trans* (Table 8). While complementation of the *sipC* deletion by plasmid-borne *sipC* was complete, complementation by *ipaC* was significantly less effective. Although the one experiment performed with SR11 would suggest that *ipaC* complementation had no effect in this strain background relative to SL1344, the three experiments performed with SL1344 also varied widely in success: the effect of *ipaC* on $SL1344^{N}_{\Delta sipC}$ invasion ranged from 0 to a 10-fold increase in bacterial invasion.

TABLE 8. Complementation of Δ*sipC* *S. typhimurium* with *ipaC* from *S. flexneri*.

Strain	Intracellular bacteria (cfu/ml)[a]
SR11	
wt	$2 \times 10^6 \pm 3 \times 10^5$
Δ*sipC*	$1 \times 10^3 \pm 6 \times 10^2$
Δ*sipC* (*sipC*$^+$)	$1 \times 10^6 \pm 4 \times 10^5$
Δ*sipC* (*ipaC*$^+$)	$2 \times 10^3 \pm 3 \times 10^2$
SL1344	
wt	$3 \times 10^5 \pm 8 \times 10^4$
Δ*sipC*	$3 \times 10^3 \pm 2 \times 10^3$
Δ*sipC* (*sipC*$^+$)	$7 \times 10^5 \pm 6 \times 10^5$
Δ*sipC* (*ipaC*$^+$)	$2 \times 10^4 \pm 1 \times 10^4$

[a]INT-407 cells were incubated with *S. typhimurium* for two hours. The infected cells were then washed to remove extracellular bacteria and incubated for a further hour in media containing 100μg/ml gentamicin. Infected cells were lysed, serially diluted and plated on LB agar plates to determine the bacterial titres. The SL1344 titres are averages ± standard deviations of three independent experiments performed in triplicate. The SR11 titres are averages ± standard deviations of one experiment performed in triplicate.

The intracellular location of Δ*sipC ipaC*-complemented *S. typhimurium* was assessed by *phoP::gfp* induction (Fig. 9). While the majority (82%) of intracellular SR11^{N} (pMLZ205) fluoresced brightly, only 12% of intracellular SR11$^{N}_{\Delta sipC}$ (pMLZ205, pWPsfc) expressed PhoP::GFP, suggesting that IpaC was indeed secreted by the *sipC*$^{-}$ strain and mediated vacuole lysis. Interestingly, DAPI staining revealed an unusual chromosomal morphology within the non-fluorescent SR11$^{N}_{\Delta sipC}$ (pMLZ205, pWPsfc) bacteria. Preparation of pMLZ205 from SR11$^{N}_{\Delta sipC}$ and re-transformation of SR11^{N} resulted in bacteria that expressed strong GFP fluorescence within INT407 cells. The lack of GFP fluorescence from intracellular SR11$^{N}_{\Delta sipC}$ was therefore not due to mutation or rearrangement of pMLZ205 within this strain. Secretion of IpaC and SipC from SR11$^{N}_{\Delta sipC}$ into the culture supernatant was confirmed by western blotting (Fig. 9, Appendix to Chapter 4); the possibility that supernatent protein was released from lysed bacteria, however, cannot be excluded since the secretion assay was not performed with a *S. typhimurium* mutant lacking a functional SP1-1 TTS system.

Intracellular conjugation experiments with Δ*sipC* recipients were performed with SR11 strains since the higher RP4 transmission frequency between SR11 parents increased the limits of transconjugant detection (Table 9). Unfortunately, SR11^{N}Δ*sipC* (*ipaC*$^{+}$) invasion was below the threshold required for detection of a significant number of transconjugant colonies. A small number of transconjugant colonies *were* recovered in these experiments. However, the frequency of these was not significantly greater than the frequency of post-plating transconjugant formation (data not shown) in the majority of experiments (the exception being expt 3, Table 9). Thus, no strong conclusions about the effect of recipient cytoplasmic relocation on the intracellular conjugation potential could be drawn.

Hypothetically, expression of IpaC within wild-type *S. typhimurium* may also result in cytoplasmic release of vacuolar bacteria without compromise of the internalization efficiency. However, *phoP::gfp* induction experiments suggested that only a small proportion of SR11^{N} (pWPsfc, pMLZ205) bacteria (15-33%) were

released into the cytoplasm (Fig. 10). That there was no difference in the RP4 transmission frequency to SR11^{N} and SR11^{N} (*ipaC*+) recipients was therefore unsurprising (Table 9). Interestingly, the same unusual chromosomal morphology was observed with SR11^{N} (pWPsfc, pMLZ205) non-fluorescing bacteria as with SR11$^{N}_{\Delta sipC}$ (pMLZ205, pWPsfc) cytoplasmic bacteria (Figs. 9 and 10).

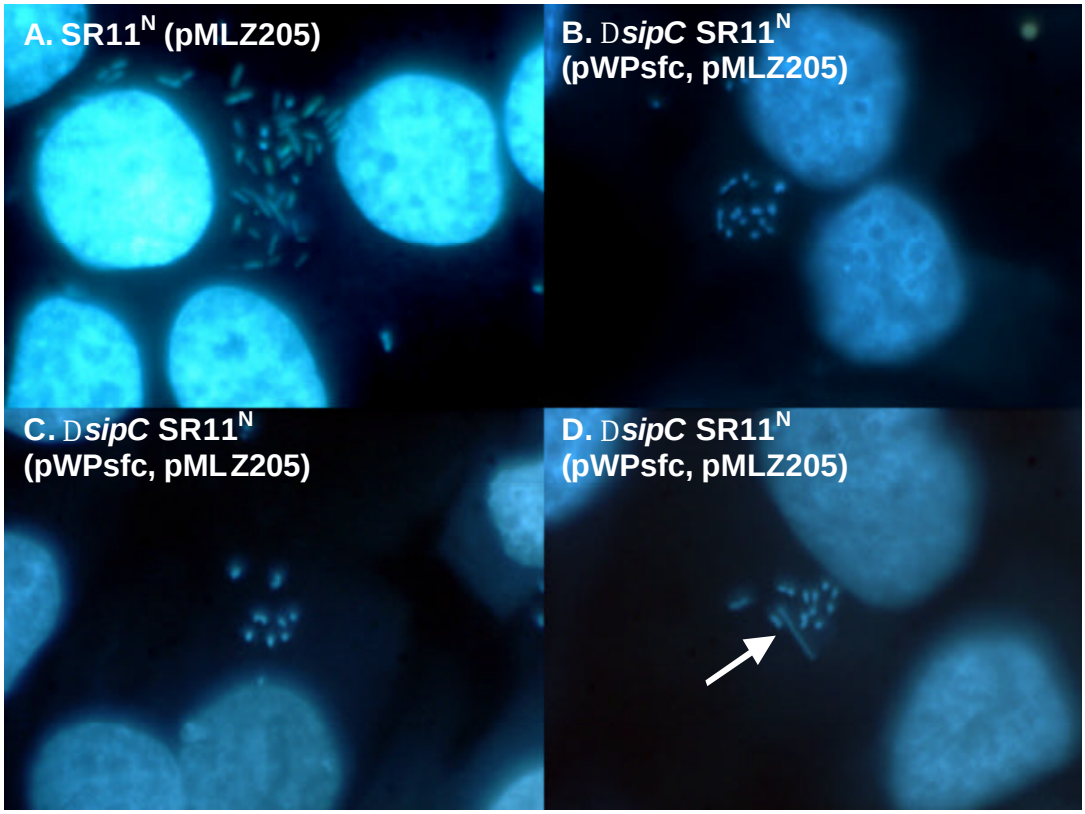

FIG.9. Intracellular D*sipC S. typhimurium* escape the vacuole. Panel A shows wild-type *S. typhimurium* stained with DAPI. Panels B, C and D show *sipC* mutant *S. typhimurium* complemented for invasion with *ipaC* on plasmid pWPsfc. *sipC* mutants did not display detectable GFP fluorescence although they carried *phoP::gfp* on pMLZ205 (data not shown). Note the unusual, 'curled up', chromosomal morphology (B, C, and D) and elongation (D, indicated by arrow) of the putative cytoplasmic bacteria compared with the wild-type morphology (A).

TABLE 9. Intracellular plasmid transmission to *ipaC*$^+$ recipients

Expt	Recipient[a]	Intracellular recipients[b]	Tranconjugant frequency[c]
1	SR11^N	$7 \times 10^5 \pm 2 \times 10^5$	$6 \times 10^{-10} \pm 2 \times 10^{-10}$ (105)[d]
	SR11$^N_{\Delta sipC}$ (*ipaC*$^+$)	$9 \times 10^3 \pm 1 \times 10^3$	1×10^{-9}; $\leq 8 \times 10^{-10}$; $\leq 1 \times 10^{-9}$ (1)
	SR11^N (*ipaC*$^+$)	$4 \times 10^4 \pm 3 \times 10^3$	$6 \times 10^{-10} \pm 2 \times 10^{-10}$ (8)
2	SR11^N	$4 \times 10^6 \pm 3 \times 10^5$	$4 \times 10^{-10} \pm 6 \times 10^{-11}$ (347)
	SR11$^N_{\Delta sipC}$ (*ipaC*$^+$)	$3 \times 10^3 \pm 3 \times 10^2$	2×10^{-9}; $\leq 2 \times 10^{-9}$; $\leq 2 \times 10^{-9}$ (1)
	SR11^N (*ipaC*$^+$)	$2 \times 10^5 \pm 3 \times 10^4$	$5 \times 10^{-10} \pm 2 \times 10^{-10}$ (16)
3	SR11^N	$1 \times 10^7 \pm 3 \times 10^6$	$2 \times 10^{-10} \pm 4 \times 10^{-11}$ (799)
	SR11$^N_{\Delta sipC}$ (*ipaC*$^+$)	$7 \times 10^5 \pm 8 \times 10^4$	2×10^{-10}; 3×10^{-10}; $\leq 1 \times 10^{-10}$ (6)
	SR11^N (*ipaC*$^+$)	$4 \times 10^4 \pm 8 \times 10^3$	$1 \times 10^{-10} \pm 4 \times 10^{-11}$ (68)
4	SR11^N	$5 \times 10^6 \pm 8 \times 10^5$	$2 \times 10^{-10} \pm 9 \times 10^{-11}$ (901)
	SR11$^N_{\Delta sipC}$ (*ipaC*$^+$)	$1 \times 10^6 \pm 4 \times 10^5$	3×10^{-10}; $\leq 1 \times 10^{-10}$; $\leq 5 \times 10^{-10}$ (6)
	SR11^N (*ipaC*$^+$)	$2 \times 10^4 \pm 4 \times 10^3$	$2 \times 10^{-10} \pm 6 \times 10^{-11}$ (147)

[a]Donors were SR11^R (RP4).

[b]Cfu/well.

[c]The plasmid transmission frequencies are expressed as the number of transconjugants divided by the *product* of the donor and recipient titres [$f(D \times R)^{-1}$]. This provided a more consistent means of comparison since the parental titres differed dramatically between the crosses within each experiment and since the conjugation potential is dependent on the invasion proficiency of *both* parents. The frequencies are averages ± standard deviations of experiments performed in triplicate.

[d]Numbers in parentheses represent the total number of transconjugant colonies.

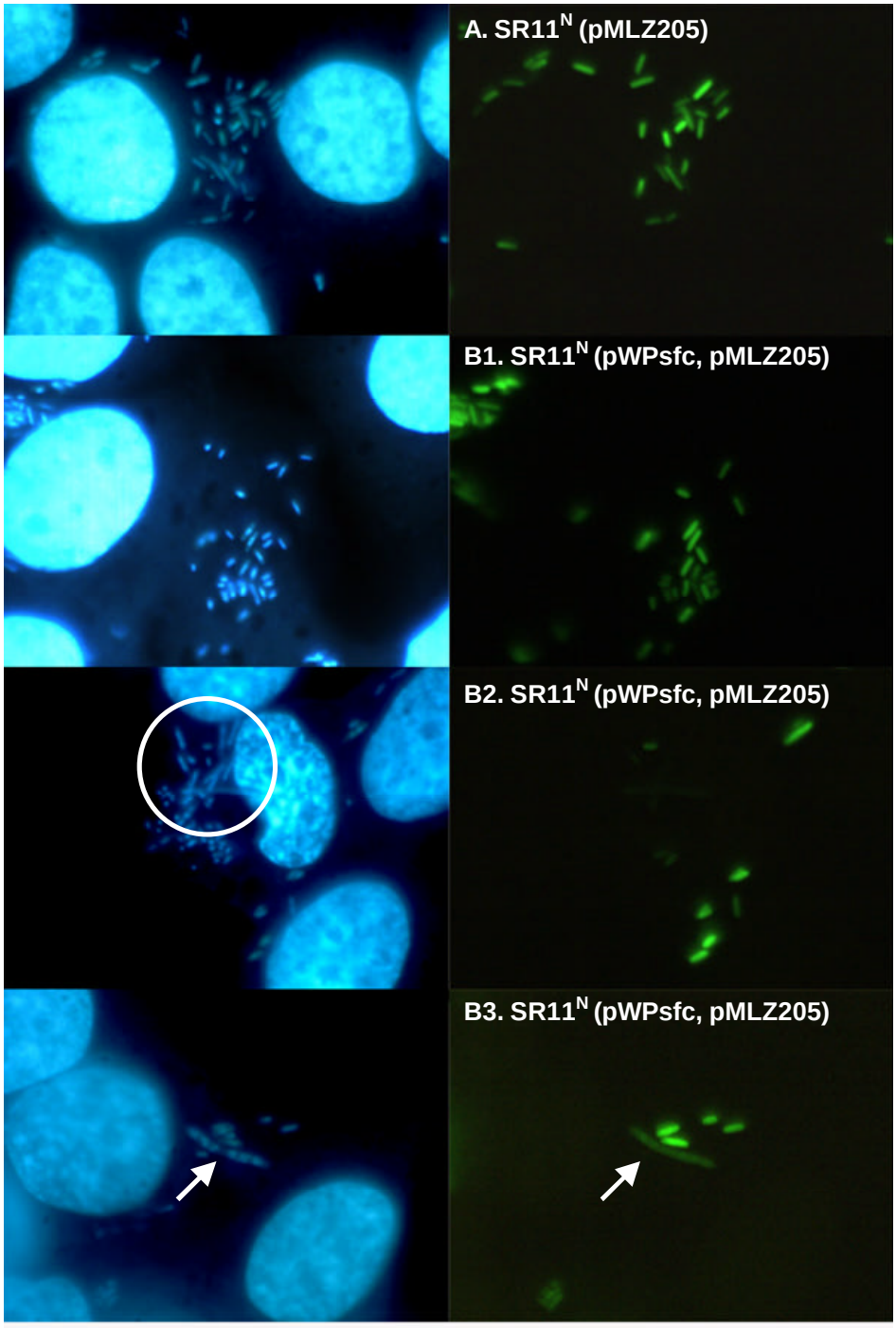

FIG.10. Only a small proportion of intracellular *S. typhimurium* relocate to the cytoplasm when expressing IpaC. Left hand panels show DAPI fluorescence and right hand panels show GFP fluorescence. Wild-type bacteria uniformly expressed *phoP::gfp* (from pMLZ205) whereas bacteria expressing IpaC (from the plasmid pWPsfc) displayed variable *phoP::gfp* induction. While some *ipaC*$^{+}$ bacteria were indistinguishable from wild-type bacteria, the cytoplasmic location of others is suggested by their elongation (ringed, B2, and indicated by an arrow, B3) and by faded or undetectable GFP fluorescence (right panels B2 and B3, indicated by an arrow).

Could intracellular conjugation be occurring between a proportion of bacteria that escape the SCV? Although no strong evidence in support of either the vacuole coalescence model or the vacuole escape model was obtained from these experiments, observation of INT-407 or MDCK cells infected with recipient *S. typhimurium* revealed that a proportion of the infected cells contained a very large number of bacteria (Figs. 7 and 8), similar to what was observed with cells infected with *sifA* mutants (Fig. 11). In one experiment, the proportion of infected cells containing an incalculably large number of bacteria was estimated at 2%. Interestingly, Brumell *et al.* reported that 1-5% of cells infected with wild-type *S. typhimurium* contained bacteria not coincident with the LAMP-1 membrane protein normally observed to co-localize with the SCV (Brumell *et al.*, 2002). These bacteria displayed characteristics similar to *sifA* mutants, namely elongation and reduced or non-expression of the *phoP::gfp* reporter. Consistent with this, the bacteria in our 'super-infected' cells generally did not express recombinant GFP from either the *phoP::gfp* or the *ssaH::gfp* vectors. Thus, it is possible that the incidence of 'super-infection' represents cytoplasmic growth of bacteria released from SCVs. Alternatively, Goetz *et al*. reported a low frequency of extensive *S. typhimurium* replication within a very small number of cells when bacteria were microinjected into the cytosol, a compartment where growth of *S. typhimurium* was not supported by the majority of host cells (Goetz *et al.*, 2001). The small proportion of permissive cells appeared to be co-incident with spontaneous cell death by apoptosis, although this correlation was not formally demonstrated.

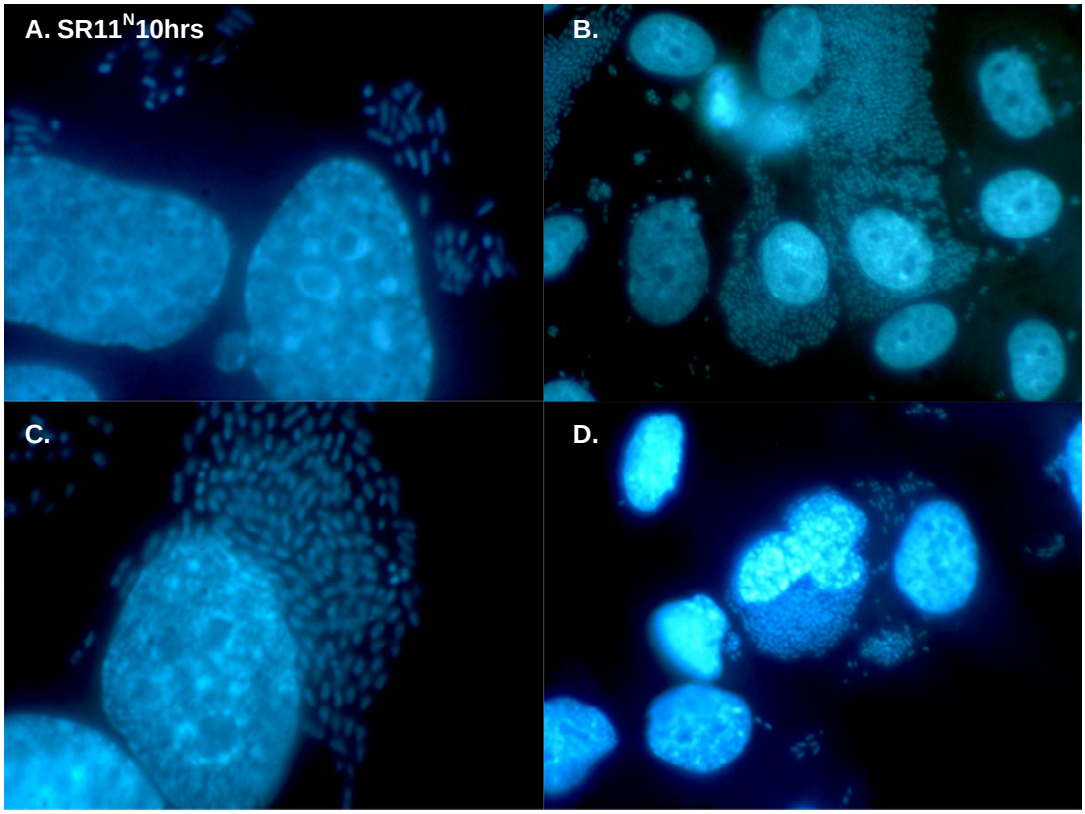

FIG.11. *sifA*⁻-like characteristics were frequently apparent within a subpopulation of wild-type recipient bacteria ten hours following entry into MDCK cells. MDCK or INT-407 cells infected with recipient *S. typhimurium* frequently contained very large numbers of bacteria when observed after the usual time-course of our intracellular conjugation experiments (B, C and D). The complete filling of the cytoplasmic space with bacteria was reminiscent of cells infected with *sifA*⁻ bacteria. Bacteria within these 'super-infected' cells generally did not express *gfp* from the plasmids pMLZ205 (*phoP::gfp*) and pFM10.1 (*ssaH::gfp*) (data not shown). However, the majority of cells infected with wild-type *S. typhimurium* contained smaller clusters of bacteria (A) which generally did express GFP from pMLZ205 and pFM10.1

DISCUSSION

The experiments presented here aimed to elucidate the means by which intracellular bacteria meet and exchange plasmid DNA. Although they shed no further light on the mechanistic particulars of intracellular conjugation, they illustrate the technical limits of a number of experimental methods in pursuit of this objective.

Initial attempts to determine the intracellular distribution of transconjugants, and hence the true permissiveness of the intracellular environment for conjugation, relied upon differential fluorescence labeling of donors and recipients (data not shown). The co-incidence of donor and recipient fluorescent markers within the same cell or vacuole was scored by epifluorescence microscopy. While GFP proved an adequate fluorescent reporter for one parental type, efforts to label the other with the red fluorescent protein reporter, DsRed (Clontech), were unsuccessful. Many report successful use of DsRed as a fluorescent reporter for *S. typhimurium* (Edwards and Maloy, 2001) and other bacteria (Tolker-Nielsen *et al.*, 2000). However, we found that expression of DsRed from a high copy number pUC-derived vector (pDs-Red, Clontech) was toxic and resulted in selection for loss of *dsRed* expression without loss of either the vector or the *dsRed* gene (data not shown). Although toxicity and instability were alleviated by expression of DsRed from a low copy vector, red fluorescence was only observed in stationary phase bacteria and only after extensive incubation at 4°C [most likely due to slow folding of the protein to its red fluorescent conformation (Baird *et al.*, 2000)]. This prevented the usefulness of DsRed in cell culture invasion experiments performed with exponentially-growing bacteria. A second red fluorescent protein, CobA (Wildt and Deuschle, 1999), and the blue fluorescent GFP variant (BFP, Clontech) displayed only low level fluorescence and rapid photobleaching and were thus of limited use as reporters.

Efforts to determine the frequency of co-infection and co-incidence ('co-clustering') of donors (GFP-labeled) and recipients (unlabeled, visualized by DAPI-staining) within cultured cells were also unfruitful. While INT-407 cells generally internalized a large number of recipients (most cells containing multiple clusters of ~5-30 bacteria), a significantly smaller number of green fluorescent donors were internalized (e.g. in one experiment only 26% of cells contained donors at 1-4 bacteria per cell). While lower donor internalization was a trend observed throughout most of the experiments presented here, and in Chapter 3, carriage of pUC vectors appeared to further decrease the efficiency of donor internalization. Thus, the number of intracellular donors in these experiments was insufficient for the detection of significant numbers of transconjugants [2]. The incidence of green fluorescent (donor) bacteria clustering amongst non-fluorescent (recipient) bacteria was very rare (e.g. one putative co-cluster within 42 infected cells) and difficult to screen for by eye. Further, a small proportion of bacteria did not display GFP fluorescence, making it impossible to count instances of co-clustering with confidence.

Consequently, two novel and complementary assays were developed in order to determine the distribution of intracellular transconjugants within cultured cells. The first was a plating assay that relied upon the enumeration of colonies resulting from the immobilization of unlysed, infected cells within a bacterial growth medium. The second was a fluorescence-based assay to report intracellular plasmid transmission by the specific induction of GFP expression within transconjugants. The plating assay enabled estimation of the proportion of cells infected with donor, recipient or recombinant bacteria. The use of flow cytometry in the fluorescence assay ideally would have facilitated the enumeration and selective sorting of cells infected with green fluorescent bacteria. Unfortunately, neither assay was successful in determining the intracellular distribution of transconjugants. Although

[2] Generally, the recovery of intracellular transconjugants at ≥ 20 colonies per plate and at a frequency at least 10-fold above the frequency of post-plating plasmid transmission was considered to be significant.

an estimate of the number of cells containing transconjugants and the average number of transconjugants per transconjugant-containing cell could be calculated from the plating assay, it was subsequently revealed that several assumptions upon which the assay was based were untrue. The titre of cell-associated colonies obtained from immobilizing infected cells in LBA supplemented with selective antibiotics appeared to underestimate the true proportion of cells infected with each *S. typhimurium* parental type. Further, the assay appeared to overestimate the number of cells containing transconjugants at the time of plating, possibly by a failure of the selective antibiotics to rapidly prevent post-plating plasmid transfer events from resulting in viable transconjugants. However, the plating assay did result in a novel observation – the 'cell-associated colony', constrained within an uncharacterized structure clearly differentiable from colonies seeded by extracellular bacteria – thus confirming the intracellular location of the recombinant bacteria recovered in these experiments (Chapter 3).

The recovery of a significant number of intracellular recombinant colonies is dependent upon high intracellular numbers of *both* parental strains. Generally, a threshold of 10^4-10^5 (per well) of the limiting parental strain was required to observe intracellular transconjugants. However, to recover intracellular recombinants at a satisfactory level of confidence, ~10^6 cfu/well of *each* parent was required. Poor internalization of SR11$^R_{ssrB::Cm}$ carrying pRK2526 and pFM10.1::Tc in the fluorescence-based intracellular conjugation assay prevented the detection of pFM10.1::Tc transconjugants, by both plating assays and FACS. The reasons for poor invasion are unknown. One possibility is that mutation of *ssrB* compromises *S. typhimurium* for invasion. Although Deiwick *et al.* report a significant reduction in expression and secretion of SPI-1 TTS gene products as a result of mutations in *ssrB* and other SPI-2 TTS genes, experimental correlation of these observations with reduced bacterial invasion was not performed (Deiwick *et al.*, 1998). In our hands, SR11$^R_{ssrB::Cm}$ invaded cultured cells with equal proficiency to SR11^R.

A second possibility is that *ssrB* mutants are compromised for survival and replication within epithelial cells. A small defect in replication within epithelial cells has been observed for *ssrB* mutants (Worley *et al.*, 2000). However, this is unlikely to be a complicating factor in our experiments since the period of time that donor bacteria reside within cultured cells prior to lysis and plating corresponds with the usual lag phase observed before initiation of wild-type *S. typhimurium* multiplication.

Interestingly, mutations in a number of SPI-2 genes, including *ssrB*, were also reported to significantly increase the susceptibility of *S. typhimurium* to complement and antibiotics such as gentamicin (Deiwick *et al.*, 1998). Thus, it is possible that prolonged incubation of infected INT-407 cells in gentamicin allows intracellular accumulation of the drug to concentrations sufficient to selectively kill $SR11^{R}_{ssrB::Cm}$. However, poor recovery of intracellular $SR11^{R}_{ssrB::Cm}$ by plating assays corresponded with direct observations of low numbers of intracellular bacteria by fluorescence microscopy. It would therefore appear most likely that compromised bacterial internalization was due to carriage of the pRK2526 and pFM10.1::Tc plasmids.

Could FACS analysis detect rare pFM10.1::Tc transfer events? In the case of the representative FACS experiment presented here, 0.53% of the negative control population [INT-407 cells infected with $SR11^{R}_{ssrB::Cm}$ (pRK2526, pFM10.1::Tc) alone] fluoresced at an intensity greater than that designated as fluorescent (Fig. 4). If the proportion of cells containing fluorescent bacteria is very small (i.e. not considerably above 0.53%), these must be of significantly greater fluorescence intensity than the background level of fluorescence to be detected as significant events. Several factors make the observation of intense fluorescence amongst pFM10.1::Tc exconjugants unlikely. Fluorescence intensity amongst the $SR11^{R}$ (pRK2526, pFM10.1::Tc)-infected cells was largely a continuum, with a small peak at a GFP fluorescence intensity of 10^3. pFM10.1::Tc exconjugants are most likely to display GFP fluorescence intensity at the lower end of the range for two

reasons. Firstly, while wild-type carriers of pFM10.1::Tc had been induced for as many as 10 hours prior to FACS, nascent recipients of pFM10.1::Tc likely have, at maximum, only a few hours for induction and accumulation of SsaH-GFP. In a *ssaH::gfp* induction time-course experiment, GFP fluorescence was first detected within a subpopulation of bacteria 1-2 hours following invasion, but GFP fluorescence intensity did not peak until 4-6 hours following invasion (data not shown). Thus, it is likely that exconjugants carrying *ssaH::gfp* display only low level fluorescence at the time that FACS analysis is performed. Secondly, while the wild-type *ssaH::gfp* carriers may harbor as many as 500 copies of pFM10.1::Tc, exconjugants may receive only one copy. Thus, *ssaH::gfp* copy number too may hinder the rate at which GFP accumulates inside transconjugants.

In addition to the technical difficulties described above, the design of this experiment may preclude the detection of one potential scenario. Upon internalization, the SCV is rapidly acidified by the action of host vacuolar proton pumps (Rathman *et al.*, 1996). Since the induction of *ssaH* via SsrAB is largely pH-dependent (Valdivia and Falkow, 1997; Cirillo *et al.*, 1998; Lee *et al.*, 2000; Valdivia and Falkow, 1996), it is likely that GFP expression would only be induced within vacuolar bacteria. Thus, the experiment may be biased towards detection of only those plasmid transmission events that occur within vacuoles. Consequently, a negative result would not allow distinction between failure to detect vacuolar transconjugants, due to low intensity fluorescence, and conjugation occurring within the cytoplasm.

Is Salmonella-specific trafficking involved in intracellular conjugation? Several of the *S. typhimurium* mutants used in this study are predicted to undergo differential trafficking within epithelial cells. Normally, phagosomes containing engulfed bacteria are targeted to the lysosomes for destruction. In contrast, trafficking of the wild-type SCV within phagocytic and nonphagocytic cells becomes uncoupled from the normal endocytic pathway (Fig. 12). SCV trafficking is determined predominantly by *S. typhimurium* genes encoded within the SPI-2 TTS system.

Entry into the endosomal environment is initially sensed by the two-component regulatory system OmpR/EnvZ. OmpR/EnvZ in turn induces transcription of a second two-component regulatory system encoded by *ssrAB* (Lee *et al.*, 2000). *ssrAB* controls the induction of SPI-2-encoded genes (e.g. *ssaH*) as well as the genes for SPI-2 secreted effectors encoded elsewhere in the *S. typhimurium* genome. One of the SPI-2 secreted effectors under SsrAB control is SifA (Beuzón *et al.*, 2000; Hansen-Wester *et al.*, 2002). SifA secretion requires a number of other SsrAB-regulated gene products, e.g. SsaH and SsaV, which form the putative "secreton" (Brumell *et al.*, 2000; Brumell *et al.*, 2001a). Therefore, mutations in other SPI-2 genes, including *ssrAB*, are predicted also to result in *S. typhimurium* vacuole escape. Contrary to prediction, *ssaV* and *ssrA* (and thus also *ssrB*) mutants are maintained within vacuoles (Beuzón *et al.*, 2000). This finding is seemingly in conflict with the proposed active role of SifA in maintenance of vacuolar integrity. However, it now appears that a second SPI-2 secreted effector protein, SseJ, is also involved in modulation of SCV membrane (Ruiz-Albert *et al.*, 2002). SseJ contributes to the loss of vacuolar membrane in the absence of SifA by an unknown mechanism.

Thus, with relevance to the experiments presented here, mutation of *ssrB* does not result in escape of bacteria from the SCV. However, *ssrB* mutation does prevent the induction and secretion of an SPI-2 gene product, SsaB, believed to mediate inhibition of SCV fusion with lysosomes (Uchiya *et al.*, 1999) [although, the precise role of SsaB in this phenomenon was recently questioned (Garvis *et al.*, 2001; Freeman *et al.*, 2002)]. Vacuoles containing *ssrB* mutants are therefore predicted to be inherently different to those that contain wild-type *S. typhimurium* (Fig. 12). However, at least one observation argues against an involvement of *Salmonella*-specific trafficking in intracellular conjugation. RP4 transmission between intracellular *E. coli* was detected at frequencies not markedly different from those detected between *S. typhimurium* (data not shown). Interestingly, when *E. coli* internalization was mediated by the *inv* gene of *Yersinia pseudotuberculosis*, plasmid transmission occurred at frequencies 10-fold higher than between *S.*

typhimurium strain SR11, in spite of relatively low numbers of intracellular *E. coli*. The reason for this is unknown but plasmid transmission occurred at wild-type frequencies when *inv* was used to mediate uptake of *S. typhimurium invA* mutants. Moreover, intracellular *E. coli* whose uptake was mediated either by wild-type *S. typhimurium* or by exogenous addition of epidermal growth factor (EGF), which stimulates endocytosis (Francis *et al.*, 1993), received and donated RP4 at equal frequencies to *S. typhimurium*. Unlike SCVs, *E. coli* vacuoles fuse with late endosomes and lysosomes (Fig. 12), thus, the particulars of vacuole trafficking appear not to affect the plasmid transmission potential. The data from the *E. coli* intracellular conjugation experiments is not presented here since the number of intracellular recombinants recovered was generally below the level of statistical significance.

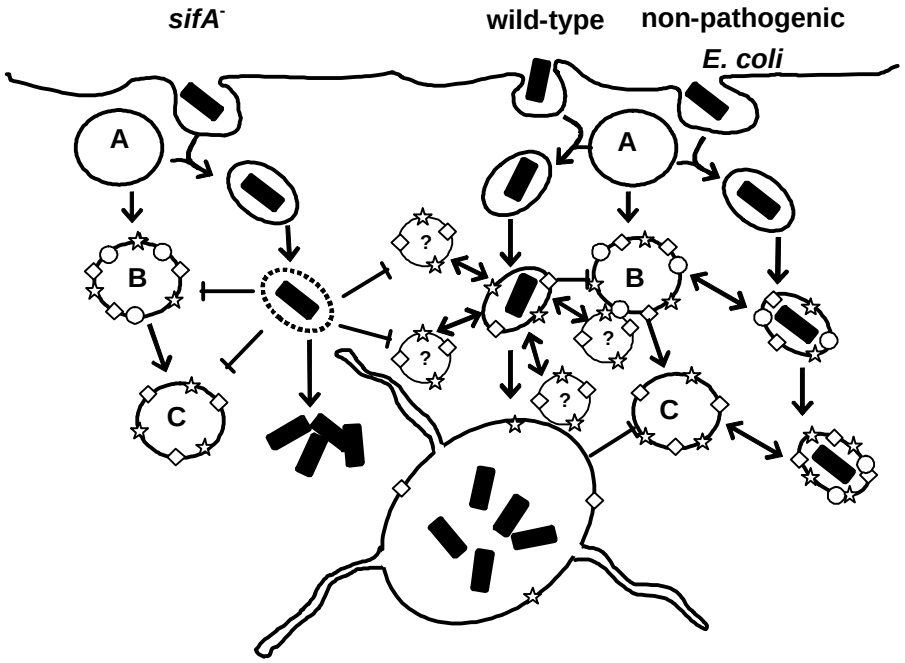

FIG.12. Model for trafficking of SCVs within non-phagocytic cells [adapted from Beuzon *et al.* (Beuzón *et al.*, 2000)]. Nascent SCVs fuse with early endosomes (A) within the first few minutes of internalization. The SCV is then rapidly acidified by the action of vacuolar proton pumps (Rathman *et al.*, 1996), the drop in pH consequently inducing acid-regulated genes such as those under the control of OmpR/EnvZ and SsrA/SsrB (Lee *et al.*, 2000; Cirillo *et al.*, 1998; Valdivia and Falkow, 1997). SCV trafficking then becomes uncoupled from the normal endocytic route. The lack of lysosomal-specific markers (e.g. the mannose-6-phosphate receptor) in the SCV membrane testifies to the failure of SCVs to fuse with late endosomes (B) and lysosomes (C), compartments dedicated to bacterial killing within phagocytic cells. Inhibition of normal trafficking is likely mediated by secretion of the SPI-2 effector protein SsaB (Uchiya *et al.*, 1999), although a recent report refutes this finding, proposing instead that *phoPQ*-regulated genes mediate SCV segregation from the normal endocytic pathway (Garvis *et al.*, 2001). In spite of its failure to fuse with lysosomes, the SCV does acquire some lysosomal-specific membrane markers, possibly via interaction with small transport vesicles (indicated by '?') (☆= vacuolar ATPase; ◊ = lysosomal glycoproteins;○ = mannose-6- phosphate receptor) (Garvis *et al.*, 2001; Rathman *et al.*, 1997; Beuzón *et al.*, 2000; Mills and Finlay, 1998; Brumell *et al.*, 2001b; Brumell *et al.*, 2001a; Méresse *et al.*, 1999; Cuellar-Mata *et al.*, 2002). Conversely, non-pathogenic bacteria like *E. coli* reside within compartments that fuse with lysosomes. Mature SCVs form sifs by the action of SifA at around 6 hours following internalization. In contrast, *sifA* mutants are unable to maintain SCV integrity by the recruitment of vesicular membrane and are consequently released into the cytoplasm.

Elucidation of the intracellular location of transconjugants and thus the means by which donor and recipient bacteria meet intracellularly proved equally elusive. Historically, vacuolar and cytoplasmic bacteria have been distinguished by their differential sensitivity to antibacterial agents such as chloroquine (Finlay and Falkow, 1988; Zychlinsky *et al.*, 1994). Chloroquine permeates cultured cells and accumulates to bactericidal concentrations within acidic compartments, selectively killing vacuolar bacteria. Addition of 2mg/ml chloroquine[3] to the cell culture medium 2 hours prior to lysis of intracellular conjugation assays resulted in an approximately equivalent reduction in the numbers of donors, recipients and transconjugants, suggesting that transconjugants were located within vacuoles (data not shown). However, an equivalent reduction in the intracellular titre of the putative cytoplasmic strain SR11$^{R}_{\Delta sipC}$ (*ipaC*$^{+}$) was also observed. Therefore, chloroquine sensitivity was deemed an unreliable indicator of intracellular location.

It was predicted that the vacuole coalescence and vacuole escape models could be distinguished if one parental type was engineered to escape the vacuole. A dramatic increase in the cytoplasmic relocation of one parent should increase the potential for cytoplasmic conjugation whilst decreasing the potential for vacuolar conjugation as the parental bacteria would be separated by a membrane. *S. typhimurium sifA*$^{-}$ mutants are reported to escape the SCV 6-10 hours following entry into cultured cells (Beuzón *et al.*, 2000; Brumell *et al.*, 2002). While some of the *sifA*$^{-}$ cytoplasmic characteristics reported by others (Brumell *et al.*, 2002) were observed here, it is likely that vacuolar escape, even after prolonged incubation, was not extensive enough for a significant change in plasmid transmission frequency to be detected. Indeed, no difference in the frequency of RP4 transmission to intracellular *sifA*$^{-}$ and wild-type *S. typhimurium* was observed. These experiments (along with the *E. coli* mating experiments discussed earlier) do, however, rule out an involvement of sifs in intracellular conjugation since *sifA*

[3] The chloroquine concentration was determined empirically as the highest concentration that did not affect bacterial growth or invasion. Chloroquine at 200μg/ml (Finlay and Falkow, 1988) did not affect intracellular bacterial titres, even after prolonged incubation, and chloroquine at 12.5-25 mg/ml (Zychlinsky *et al.*, 1994) was cytotoxic.

mutants fail to mediate the formation of the tubular vacuole membrane filaments characteristic of wild-type SCVs (Stein *et al.*, 1996).

Consequently, a second vacuole-escaping mutant of *S. typhimurium* was engineered. The property of vacuole lysis can be conferred upon *S. typhimurium* by substitution of its SPI-1 TTS system effector protein, SipC, with the *S. flexneri* homologue, IpaC (Osiecki *et al.*, 2001). Entry of *S. typhimurium* and *S. flexneri* into cultured cells is mediated by homologous TTS systems encoded, respectively, on SPI-1 (*inv*/*spa*) and the 220Kb virulence plasmid (*ipa*/*mxi*/*spa*). The protein products of the TSS systems form a bacterial membrane-spanning “needle complex” which extends outwards into the external milieu (Kubori and Galán, 2002; Ginocchio *et al.*, 1994; Magdalena *et al.*, 2002; Kubori *et al.*, 1998). The needle complex is believed to form a channel through which effector proteins are secreted upon contact with the target animal cell (Kubori and Galán, 2002; Magdalena *et al.*, 2002; Kubori *et al.*, 1998). Since the exogenous addition of SipC and IpaC to cultured cells mediates cytoskeletal rearrangements and internalization of extracellular bacteria (Terajima *et al.*, 1999; Osiecki *et al.*, 2001; Picking *et al.*, 2001; Kuwae *et al.*, 2001; Tran *et al.*, 2000), it is likely that effector proteins enter host cell membranes and cytoplasms extracellularly rather than by direct injection into the host cell cytoplasm by the needle complex. In contrast to the TTS apparatus, the effector proteins are poorly conserved between intracellular pathogens and diverse in function. Never-the-less, reciprocal complementation of some *S. flexneri* and *S. typhimurium* effectors has been reported (Galán, 1996; Groisman and Ochman, 1993; Hermant *et al.*, 1995).

Expression of the *S. flexneri* gene *ipaC* within an *S. typhimurium sipC* mutant appeared to mediate vacuolar escape (evidenced by a failure to display GFP fluorescence when carrying the *phoP::gfp* reporter construct). However, IpaC failed to convincingly complement *sipC* mutants for internalization. In contrast, others report that IpaC complemented a *S. typhimurium sipC* mutant to 45% of the level of wild-type invasion (Osiecki *et al.*, 2001). The reason for this difference is unknown

but is unlikely to be accounted for by differences between the *sipC* transposon mutant used in Osiecki *et al.*'s study (Kaniga *et al.*, 1995; Osiecki *et al.*, 2001) and the *sipC* deletion mutant created in this study. Deletion of *sipC* did not affect expression of the downstream gene *sipD* since complementation with *sipC in trans* restored the invasive phenotype. Furthermore, both *sipC* mutant strains were equally complemented by *ipaC* when invasion experiments were performed in another laboratory (Wendy Picking, personal communication).

The failure of IpaC to mediate *sipC*$^{-}$ *S. typhimurium* internalization above the required threshold prevented test of the vacuolar coalescence model. Thus, the subcellular location of *S. typhimurium* conjugants remains undetermined.

An interesting (and as yet unresolved) question is whether the ability for bacteria to replicate in the nutrient-rich cytoplasm requires specific adaptation (Goebel and Kreft, 1997; O'Riordan and Portnoy, 2002). That *S. typhimurium* (and other vacuolar pathogens) failed to replicate when microinjected directly into the cytosol supports the contention that the cytosol is not generally replication-permissive (Goetz *et al.*, 2001). However, others have demonstrated cytoplasmic replication of both vacuolar pathogens and non-pathogenic *E. coli* when engineered to escape into this compartment (reviewed by O'Riordan and Portnoy, 2002). Pertinent to this work, *sifA S. typhimurium* mutants appeared to replicate extensively in the cytoplasm (herein and Brumell *et al.*, 2002). In contrast, *S. typhimurium sipC* mutants escaping the vacuole by virtue of IpaC expression did not appear to replicate within the cytoplasm (Wendy Picking, personal communication). It is possible that pre-adaptive events that occur whilst *S. typhimurium* reside within the SCV are required to prime them for cytoplasmic growth (Brumell *et al.*, 2002). Alternatively, the cytosols of different cell lines may not be equally permissive for bacterial replication.

In summary, a variety of technical difficulties prevented practical test of the intracellular conjugation models discussed within this chapter. Although no support

for either the vacuole coalescence or vacuole escape model was derived from the experiments attempted here, it is tempting to speculate that intracellular conjugation occurs within the small proportion of infected cells that contain incalculable numbers of potentially cytoplasmic bacteria. Certainly, the filling of host cell cytoplasms with rapidly-dividing bacteria would promote bacterial contact and conjugative plasmid transmission. It is as yet unclear whether low frequency SCV deterioration is a result of host cell or bacterial plasticity.

That intracellular conjugation occurs equally efficiently between intracellular *E. coli* and between *sifA*$^{-}$ *S. typhimurium* suggests that aspects of vacuole trafficking specific to the SCV are not involved in intracellular mixing of donors and recipients. However, since the calculated *E. coli* intracellular plasmid transmission frequencies were based upon insignificant numbers of transconjugant colonies, this assertion requires verification by other means. Potentially, the role of specific SCV trafficking in intracellular conjugation could be tested by drug-mediated or genetic inhibition of normal SCV trafficking within the host cell. For example, addition of the drug wortmannin to cultured cells prevents many cellular endocytic and secretory processes (Brumell *et al.*, 2002). Furthermore, transfected cell lines expressing dominant negative mutants of Rab5 and Rab7 GTPases are disabled for early- and late- endosomal trafficking events, respectively (Méresse *et al.*, 1999; Brumell *et al.*, 2002). A combination of drug inhibition and Rab5/Rab7 mutant cell lines may be useful in testing the involvement of vacuolar fusion events in intracellular conjugation.

Also of interest is whether host cell and bacterial *de novo* protein synthesis is required for intracellular plasmid transmission. Host cell protein synthesis is inhibited by the drug cyclohexamide (e.g. Allen *et al.*, 2000) and bacterial protein synthesis can be inhibited by the host cell-permeating antibiotics tetracycline and chloramphenicol (Deiwick *et al.*, 1998; Allen *et al.*, 2000) without concomitant decreases in conjugative plasmid transfer or transmission (Cooper and Heinemann, 2000).

Ultimately, test of the models for intracellular conjugation proposed herein could be performed by engineer of a conditional plasmid transfer system. A conjugative plasmid deleted for an essential mobilization gene could be complemented for transfer by a *trans*-acting mobilization protein whose conditional expression was dependent upon intracellular induction. For example, expression of *mob* functions from the *phoPQ* or *ssrAB* promoters would detect only plasmid transmission occurring within vacuoles. Conversely, cytoplasmic plasmid transmission could be distinguished by expression of *mob* functions from the *Listeria monocytogenes* cytoplasmically-induced *actA* promoter (Goetz *et al.*, 2001).

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Chapter 5: The Putative Conjugative Escort Proteins TraI and MobA Localize to Human Cell Nuclei*

ABSTRACT

Conjugation is probably the most universal of HGT mechanisms, mediating gene transfer not only between bacterial species but also from bacteria to yeast, plant and even animal cells. DNA transferred between bacteria and eukaryotic organisms (trans-kingdom conjugation) faces the further challenge of traversing the eukaryotic cell nuclear membranes before the transferred genes can be expressed and inherited. Trans-kingdom gene transfer conducted by the Ti plasmid of *Agrobacterium tumefaciens* probably involves a protein-DNA complex. The VirD2 protein of this complex contains nuclear localization signals that might contribute to the transport of T-DNA to the plant nucleus. While *Agrobacterium* has evolved specialized means of efficient DNA transfer to the plant cells with which it interacts, the means by which transferred DNA traverses recipient cell nuclei during trans-kingdom conjugation between non co-evolved organisms remains biochemically unexplained. What is the involvement, if any, of proteins in mediating conjugative gene transfer to eukaryotic cells in general? Is nuclear translocation a limiting step in conjugative transfer to eukaryotic cells? To address these questions I looked for nuclear localizing potential inherent within the putative escort proteins TraI and MobA from the broad host-range bacterial plasmids RP4 and RSF1010, respectively. Translational fusions of TraI and MobA polypeptides to EGFP were expressed within cultured human cells (INT-407). TraI showed strong localization to human cell nuclei and nuclear localization was found to be only partially attributable to a putative bipartite nuclear localization sequence (NLS) predicted by protein sequence analysis. In contrast, the full-length MobA protein, did not exclusively accumulate in the nucleus. Although by no means comprehensive, these results suggest the possibility that the requirement for nuclear localization potential within the putative escort proteins of trans-kingdom conjugation may be universal. However, *efficient* nuclear permeation may not be an absolute necessity for trans-kingdom gene transmission by conjugation.

* Manuscript in preparation.

INTRODUCTION

The previous experimental chapters of this thesis have been concerned with the transfer of DNA between bacteria residing within cultured human cells. This chapter is concerned with the transfer of DNA by conjugation between bacteria and eukaryotic cells ('trans-kingdom conjugation'). Trans-kingdom conjugation was reviewed in chapter one. Here, gene transfer from bacteria to eukaryotic cells will be discussed with particular attention to the role of conjugative proteins in mediating translocation of DNA across nuclear membranes.

Bacterial conjugation is not merely a phenomenon restricted to the exchange of genes between bacteria. Conjugation is now recognized as a broadly relevant gene transfer mechanism, mediating gene transfer both between bacterial species and from bacteria to yeast, plant and even animal cells (for representative examples see Beijersbergen *et al.*, 1992; Bundock *et al.*, 1995; Heinemann and Sprague Jr, 1989; Kunik *et al.*, 2001; Waters, 2001). An unanswered question, therefore, is: how does DNA that is transferred to eukaryotic cytoplasms during conjugation traverse the nuclear membrane to bring about expression or inheritance?

As discussed previously (see Chapter One), it is likely that the cell wall itself poses little barrier to gene transfer by conjugation; conjugative gene transfer from gram negative to gram positive bacteria, yeast, plant and animal cells demonstrates that there are few, if any, requirements for a cell to be a recipient (Beijersbergen *et al.*, 1992; Heinemann and Sprague Jr, 1989; Kunik *et al.*, 2001; Waters, 2001). Thus, the barriers to gene transmission (as opposed to transfer) must explain why gene transfer between certain partners is not readily detected in the environment. Transmission barriers include degradation of transferred DNA by recipient restriction enzymes, failure of transferred DNA to replicate, segregate or integrate and failure of the incoming genes to be expressed when gene expression is necessary for inheritance (Heinemann, 1991). When transferred to eukaryotes,

DNA encounters a further barrier if it is to be expressed or inherited. Unlike prokaryotic organisms, eukaryotic organisms separate DNA replication and transcription from cytoplasmic processes, such as translation of mRNA transcripts, by containing their chromosomal DNA within a double membrane-bound nucleus (Nigg, 1997).

The nuclear membranes appear not to pose an impenetrable barrier to DNA since DNA that is introduced to the cytoplasm by microinjection, electroporation, lysis of intracellular bacteria or other means penetrates the nucleus and is expressed (for examples see Courvalin *et al.*, 1995; Grillot-Courvalin *et al.*, 1998; Bundock *et al.*, 1995; Sizemore *et al.*, 1995; Zupan *et al.*, 1996; Bongartz *et al.*, 1994; Sizemore *et al.*, 1997). However, in the most extensively studied example of trans-kingdom conjugation, the T-DNA transfer system of *A. tumefaciens*, evidence strongly supports the concept that T-DNA is transferred (or reconstituted *in planta*) as a protein-DNA complex, somewhat larger than that which could reasonably be expected to traverse the nuclear membranes by passive diffusion (Citovsky *et al.*, 1997). It is argued that T-DNA itself does not encode a nuclear localizing sequence since the entire sequence of DNA between the delimiting T-DNA borders can be replaced without concurrent reduction of transmission frequency (Zambryski, 1988). Thus, if the T-DNA is transferred to the plant cell nucleus as a protein-DNA complex it is necessary to evoke the idea of nuclear localizing ability being inherent within the proteins that are transported with the DNA. But what evidence is there that proteins are transferred along with DNA during conjugation?

Protein transfer during conjugation

Evidence for protein transfer during conjugation is predominantly indirect. A convincing line of evidence for the ability (if not the actuality) of protein transfer during conjugation is the discovery that proteins can transfer from bacteria to eukaryotic cells by a mechanism *resembling* conjugation (Vogel *et al.*, 1998; Segal *et al.*, 1999; Segal and Shuman, 1999; Segal *et al.*, 1998; Ferguson and

Heinemann, 2002; Llosa *et al.*, 2002). The role of type IV secretion of proteins in pathogenesis and the relationship of protein transfer systems to DNA transfer systems was reviewed in Chapter One and so will not be revisited in depth here. Briefly, protein transfer systems dedicated to virulence likely evolved from (or alongside) DNA transfer systems (Winans *et al.*, 1996; Ferguson and Heinemann, 2002; Christie, 2001; Christie and Vogel, 2000) and are in some cases similar enough to be interchangeable (Vogel *et al.*, 1998; Segal *et al.*, 1999; Segal *et al.*, 1998) whereas in others, competitive (Binns *et al.*, 1995; Stahl *et al.*, 1998; Segal and Shuman, 1998). Since the substrates translocated by the type IV secretion systems of intracellular pathogens are most likely proteins and not DNA, it seems reasonable to suppose that systems dedicated to the transfer of DNA too act upon proteins as the significant component of the translocated substrate (Llosa *et al.*, 2002).

Speculation on conjugative protein transfer from donor to recipient cells is supported by some physical evidence of both DNA-associated and non DNA-associated protein transfer during conjugation (Heinemann, 1999; Rees and Wilkins, 1989; 1990; Weld and Heinemann, 2002; Wilkins and Thomas, 2000). Furthermore, the role of proteins in the transmission of DNA, as protein-DNA complexes, is particularly well described for *Agrobacterium tumefaciens* (for a review see Christie, 2000).

Ti conjugative proteins are transferred to plant cells with and without T-DNA.

The analogy of T-DNA transfer to bacterial conjugation is now universally accepted (for a review see Chapter One and Lessl and Lanka, 1994). To recapitulate, DNA transfer is initiated when VirD2 nicks the lower strand of the Ti plasmid at the T-DNA delimiting right border. VirD2 becomes covalently attached (at Tyr_{29}) to the 5' end of the nicked DNA. A second nicking reaction takes place at the left border to release a single-stranded linear molecule, the T-DNA. T-DNA is transferred to the plant cell nucleus in this single-stranded linear form (Yusibov *et al.*, 1994; Tinland

et al., 1994) likely bound at the 5' end by VirD2 and potentially bound along its length by the single-stranded DNA binding protein VirE2.

That VirD2 accompanies T-DNA on its journey from the bacterial cytoplasm to the plant cell nucleus is supported indirectly by its covalent attachment to transfer intermediates (Pansegrau *et al.*, 1993a) and by the necessity of VirD2 for precise (5' conserved) integration of T-DNA into the plant genome (Tinland *et al.*, 1995). However, the case of VirE2 provides the strongest evidence for conjugative protein translocation (Vergunst *et al.*, 2000). VirE2 is necessary for tumorigenesis following T-DNA transfer to plant cells but is not necessary for T-DNA transfer *per se*: a similar quantity of ssT-DNA was recovered from plant cells following incubation with *virE2* mutants as with *Agrobacterium* carrying wild-type Ti (Yusibov *et al.*, 1994) and *virE2* mutants can be complemented extracellularly for tumorigenicity either by a second VirE2-expressing strain (Otten *et al.*, 1984; Binns *et al.*, 1995) or by expression of VirE2 within transgenic plant cells (Citovsky *et al.*, 1992). Thus, the contribution of VirE2 to T-DNA transmission takes place *in planta* and VirE2 can be translocated independently of T-DNA (Simone *et al.*, 2001). Extracellular complementation of *virE2* mutants with VirE2 donors is dependent on the *virB* genes and attachment of donor bacteria to plant cells (Christie *et al.*, 1988; Binns *et al.*, 1995). Thus, VirE2 translocation likely occurs by the same mechanism as transfer of the T-complex. Since T-DNA transferred to plant cells in the absence of VirE2 reaches the nucleus inefficiently and is integrated with large 3' truncations (Rossi *et al.*, 1996), VirE2's likely *in planta* role is protection of T-DNA from nucleolytic attack with the possibility of a secondary involvement in import of the T-complex to the nucleus.

Are VirD2 and VirE2 involved in nuclear import of T-DNA?

In addition to their T-DNA processing and protection functions, VirD2 and VirE2 may play a role in import of the T-complex into plant cell nuclei. In general, proteins with known nuclear function possess at least one of two consensus sequences (for

a review see Dingwall and Laskey, 1991). The first is a short, monopartite sequence of basic amino acid residues typified by that of the SV40 large T antigen. The second is bipartite, comprising two basic motifs separated by a non-conserved spacer of roughly 10 amino acids, the prototype being the nucleoplasmin NLS of *Xenopus laevis*.

VirD2 localizes to plant cell nuclei

VirD2 has two potential NLS's; an N-terminal monopartite and a C-terminal bipartite consensus sequence (Shurvinton *et al.*, 1992; Tinland *et al.*, 1992; Howard *et al.*, 1992). Interestingly, while the C-terminal region, which is not necessary for DNA processing, is <20% conserved between VirD2 homologues from the Ti plasmid family as a whole, the putative NLS motif within this region is highly conserved, implying that this sequence is involved in an essential function (Tinland *et al.*, 1992).

There is experimental evidence for NLS activity. VirD2 localizes to the nucleus when expressed in plant cells as a GUS-VirD2 fusion. Deletion of the bipartite NLS renders GUS-VirD2 cytoplasmic (Howard *et al.*, 1992). The functionality of the second, monopartite, NLS is controversial (Relic *et al.*, 1998; Tinland *et al.*, 1992; Shurvinton *et al.*, 1992; Howard *et al.*, 1992; Ziemienowicz *et al.*, 2001). The nuclear-localizing ability of VirD2 was linked to its role in T-DNA transmission by the observation that NLS deletions that rendered VirD2 cytoplasmic also reduced tumorgenicity, however, the extent of the attenuation appeared to be plant tissue-specific (Shurvinton *et al.*, 1992). Although the effect of C-terminal deletions on the other functions of VirD2 were not assessed, it is unlikely that deletion of the NLS affected DNA processing in the bacterium or integration *in planta* (Ziemienowicz *et al.*, 1999; Bundock *et al.*, 1995; Tinland *et al.*, 1995).

VirE2 also localizes to plant cell nuclei

VirE2 also appears to have inherent nuclear localizing ability: GUS-VirE2 fusion proteins localized to the nucleus when expressed in plant cells (Citovsky *et al.*, 1992) and, similarly to VirD2, deletion of putative bipartite nuclear localization sequences in the central region of VirE2 rendered GUS-VirE2 cytoplasmic. The VirE2 sequence contains two bipartite NLS's, NSE-1 and NSE-2, but they are poor matches with imperfect similarity to the nucleoplasmin consensus (Citovsky *et al.*, 1992). Deletion of NSE-1 causes a small decrease in nuclear localization, as does deletion of NSE-2. Deletion of both regions abolishes nuclear localization.

Is VirE2 nuclear localizing ability involved in T-DNA nuclear translocation?

To date it has not been possible to distinguish between two non-mutually exclusive models for the role of VirE2 in T-complex nuclear translocation. It is proposed that the initial targeting of the T-complex to the plant proteins that mediate nuclear import is achieved by VirD2 (Citovsky *et al.*, 1992). This is supported by the observation that VirE2's nuclear localization ability does not account for the residual T-DNA transmission from an *A. tumefaciens* Ti VirD2$_{\Delta NLS}$ mutant (Rossi *et al.*, 1996) and that VirE2 alone is not sufficient to direct ssDNA to the nucleus of plant or animal cells (Ziemienowicz *et al.*, 1999; Ziemienowicz *et al.*, 2001). Translocation of an *in vitro* constituted T-complex to HeLa nuclei was dependent on both the VirD2 NLS and VirE2. Thus, VirE2 must play a secondary role in translocation of the T-complex. Unfortunately, problems arise in determining this secondary role of VirE2 due to the inseparability of VirE2's nuclear localizing ability and its single-stranded DNA-binding ability (Citovsky *et al.*, 1992). NSE-1 deletants do not bind ssDNA at all and NSE-2 deletants are partially compromised for ssDNA binding. Either VirE2 aids translocation directly, by interacting with the nuclear import machinery (Citovsky *et al.*, 1992), or indirectly, by maintaining the rigid unfolded structure of the T-DNA complex during import (Rossi *et al.*, 1996; Citovsky *et al.*, 1997). In the latter case, the nuclear localizing ability of VirE2 would be merely fortuitous.

An indirect role for VirE2 in nuclear translocation of the T-complex is favoured by Ziemienowicz *et al.* (Ziemienowicz *et al.*, 2001; Ziemienowicz *et al.*, 1999). VirE2 alone was unable to mediate the nuclear import of *in vitro* assembled ssDNA complexes. Rather, the association of VirE2 with ssDNA *prevented* nuclear import of the protein (Ziemienowicz *et al.*, 2001). Thus, it would appear that ssDNA binding results in sequestration of VirE2's nuclear localization domains. VirE2 was necessary for nuclear import of *in vitro*-assembled T-complexes, however, the ssDNA-binding protein RecA was able to mediate efficient nuclear import of the complex in place of VirE2. Interestingly, RecA expressed *in planta* failed to complement an *A. tumefaciens* Ti *virE2* mutant for T-DNA transmission, suggesting that VirE2 performs a specific function either prior to or following nuclear import of the T-complex. Although an interaction between VirE2 and the nuclear import machinery cannot be ruled out, this is unlikely to involve the nuclear localization ability demonstrated for the unbound VirE2 protein.

However, it should be noted that Zupan *et al*. *did* observe VirE2-ssDNA localization to plant nuclei (Zupan *et al.*, 1996) and a direct involvement of VirE2's nuclear localization functions in nuclear import of the T-complex is supported by others (Tzfira *et al.*, 2001; Citovsky *et al.*, 1994).

Citovsky *et al.* circumvented the problem of VirE2's inseparable functions to a certain extent by an experiment that did not measure T-DNA transmission from VirE2 mutants directly but instead measured the effect of competing NLS motifs on wild-type T-DNA transmission (Citovsky *et al.*, 1994). The experiment was founded on the underlying assumption that if NSE-1 and -2 are involved in T-DNA nuclear import, then T-DNA nuclear translocation should be inhibited by interaction of those same motifs, within competing proteins, with the nuclear import machinery (Citovsky *et al.*, 1994). Indeed, small decreases in tumorigenicity were observed when plant cells expressed wild-type VirE2 or VirE2$_{\Delta NSE-1}$ whereas large decreases in tumorigenicity were observed when plant cells expressed VirE2$_{\Delta NSE-2}$ or a VirE2 mutant deleted only for its DNA binding function (Citovsky *et al.*, 1994). The lesser

effect on tumorigenicity of VirE2 proteins retaining their DNA binding function could be interpreted in two ways: either these proteins are sequestered by binding to mRNA, or the dramatically attenuating mutant forms of VirE2 interact irreversibly (as opposed to wild-type VirE2 interacting reversibly) with the plant factors that mediate nuclear localization (Citovsky *et al.*, 1994). However, the possibility that the *in planta*-expressed VirE2 variants interfered in other ways with T-DNA transmission was not excluded. Unfortunately, *Agrobacterium* crosses with plant cells expressing a $VirE2_{\Delta NSE\text{-}1.2}$ double mutant protein, which would be predicted to neither interfere with nuclear localization nor be sequestered by mRNA, were not performed. Further, if binding of VirE2 to nucleic acids renders it ineffective in nuclear import, this would argue against a biological role for VirE2 in the nuclear import of T-DNA as suggested by Ziemienowicz *et al.* (Ziemienowicz *et al.*, 2001). The above results could as easily be explained by interference of over-expressed, unbound VirE2 proteins with VirD2-mediated nuclear import of T-DNA. Despite this, a model for VirE2's involvement in nuclear translocation of the T-complex endures.

Plant proteins mediate nuclear import of the T-complex via interaction with VirD2 and VirE2

Perhaps the best evidence for necessity of VirD2 and VirE2 nuclear localizing ability for T-DNA transmission comes from studies that have sought the intermediary plant nuclear import factors since such studies do not reply upon mutation of VirD2 and VirE2. Briefly, proteins that carry an NLS are translocated to the nucleus through 'nuclear pore complexes' (NPCs), complex cylindrical arrangements of proteins that span the nuclear double membrane (for reviews see Nigg, 1997; Spector, 1993). While proteins below a certain size (approximately 45kDa) are believed to traverse the nucleus by passive diffusion, larger proteins must do so by first interacting with NLS receptor proteins, which mediate the interaction with the NPC and ferry the protein cargo across to the nucleus. Such interactions are mediated by the NLS within the imported protein. Although

alternative transporters may exist, the majority of nuclear proteins in animal cells enter the nucleus by interaction with the cytoplasmic protein importin-α. Importin-α then forms a heterodimer with importin-β, which docks the complex to the NPC. Translocation of the cargo-carrier complex requires energy in the form of GTP hydrolysis by the cytoplasmic protein Ran-GTP. Following translocation, nuclear importin-α and -β are recycled back to the cytoplasm by an unknown mechanism.

An NLS receptor protein that interacts with VirD2 has been isolated. The *Arabadopsis thaliana* protein AtKAPα interacts specifically with VirD2 but not VirD2$_{\Delta NLS}$ or VirE2 and is homologous to importin-α and the yeast SRP1 proteins (Ballas and Citovsky, 1997). Since plant genetics to date lacks a clean system for knock-out of specific plant genes, experiments to elucidate the role of AtKAPα in VirD2 nuclear import were carried out in yeast. AtKAPα was found to complement a yeast *srp1-31* temperature-sensitive mutant for growth at the restrictive temperature, indicating the interchangeability of plant and yeast nuclear import machinery. Yeast cytosolic fractions prepared at the restrictive temperature complemented VirD2 for import to the nucleus of permeabilized yeast cells only when the cytosolic fraction contained either AtKAPα or SRP1 and only when VirD2 carried an NLS. Nuclear import of VirD2 was blocked by synthetic peptides corresponding to the NLS, presumably by competition for AtKAPα-binding. A necessity for AtKAPα in T-complex nuclear import awaits rigorous testing, however, since mating experiments between *A. tumefaciens* and *srp1-31* yeast have yet to be performed.

The story is more complete for VirE2. The *Arabidopsis* protein VIP1 was isolated similarly on account of its interaction with VirE2 (Tzfira *et al.*, 2001). However, in contrast to AtKAPα, VIP1 is not an importin-α homologue, but a basic zipper protein (bZip) with its own bipartite NLS. As predicted, VIP1 localized to the nucleus of both yeast and plant cells. The NLS-like sequences of VirE2 fail to mediate nuclear uptake in yeast, but VirE2 could be complemented for nuclear

localization by co-expression with VIP1. T-complex transfer to the nucleus appeared to be dependent on VIP1 since transgenic tobacco lines expressing VIP1 antisense mRNA (such that the levels of sense mRNA were greatly decreased) showed a dramatic decrease in tumorigenicity when subjected to *Agrobacterium* infection. GUS-VirE2 localization within the antisense plants was shown to be cytoplasmic whereas the nuclear localization of GUS-VirD2 and GUS-VIP1 was independent of VIP1. Thus, it appears that VirE2 nuclear import occurs by a mechanism independent of that of VirD2, involving “piggy-back” association with VIP1. No non-plant homologues of VIP1 have been identified, which may explain why VirE2 nuclear localization has not been observed in yeast and animal systems (Guralnick *et al.*, 1996; Tzfira *et al.*, 2001), although, this assertion is disputable since Ziemienowicz *et al.* reported VirE2 localization to the nuclei of cultured human cells (Ziemienowicz *et al.*, 1999). Compounds that interfere with the NPC inhibited nuclear import of VirE2-DNA complexes (Zupan *et al.*, 1996) and the prototypical nuclear import pathway was subsequently shown to be responsible for VIP1-VirE2 import (Tzfira *et al.*, 2002).

While it appears likely that VirE2 plays some role in the nuclear import of the T-complex [in addition to its role in protection of T-DNA from degradation in the plant cell cytoplasm (Rossi *et al.*, 1996)], it is important to note that T-DNA transmission is observed in Agrobacterium x *Saccharomyces cerevisiae* crosses in spite of an inability of VirE2 to localize to yeast nuclei (Bundock *et al.*, 1995; Tzfira *et al.*, 2001).

Trans-kingdom conjugation between non co-evolved organisms

It is not particularly surprising that *Agrobacterium* has evolved means of efficient conjugative plasmid transfer to the plant cells with which they interact. Perhaps of greater surprise are the instances of trans-kingdom conjugation shown to occur between non co-evolved organisms. What is the involvement, if any, of proteins in mediating conjugative gene transfer to eukaryotic cells in general? If it is accepted

that conjugation proceeds by transfer of a ssDNA molecule covalently bound at the 5' end to a protein, then can other conjugative proteins mediate nuclear transformation? Is nuclear translocation a limiting step in conjugative transfer to eukaryotic cells?

The nuclear localization ability of Ti conjugative proteins is also involved in import of T-DNA to yeast and animal cell nuclei

In the case of Ti, the nuclear localizing ability of its conjugation proteins and the conservation of nuclear import pathways between diverse eukaryotic organisms may serve to explain successful T-DNA transfer to non co-evolved organisms (Bundock *et al.*, 1995; Kunik *et al.*, 2001). The NLS of VirD2 was shown to be functional in *Drosophila melanogaster* embryos, *X. laevis* oocytes (Guralnick *et al.*, 1996), the yeast *S. cerevisiae* (Tzfira *et al.*, 2001) and cultured human cells (Relic *et al.*, 1998). Correspondingly, conjugative T-DNA transfer to *S. cerevisae* and to cultured human cells does occur, albeit at a low frequency (Bundock *et al.*, 1995; Bundock and Hooykaas, 1996; Piers *et al.*, 1996; Kunik *et al.*, 2001) (see Chapter One). Pertinent to this discussion, T-DNA transmission to yeast was dependent on the C-terminal region of VirD2 (encoding the NLS). Thus, the requirement for nuclear localization signals within the T-complex is unlikely to be limited to plant cells.

Do 'traditional' conjugative proteins also mediate transfer of plasmid DNA across nuclear membranes?

More interesting are the cases of conjugative DNA transmission mediated by proteins not expected to have evolved nuclear localizing ability. These include MobA of the IncQ plasmid family, TraI of the IncPα family and TraI of the F plasmid family, the functional homologues of VirD2 (Pansegrau *et al.*, 1993b; Bhattacharjee and Meyer, 1993; Pansegrau *et al.*, 1993a) (see Chapter One). Since TraI and MobA are involved in conjugation systems encoded on plasmids not known to be

involved in any naturally occurring eukaryotic DNA transfer phenomena, there is no *a priori* expectation that these proteins contain any inherent nuclear localizing ability. Never-the-less, MobA and TraI mediate conjugative transmission to nucleated organisms at a comparatively high frequency (Heinemann and Sprague Jr, 1989; Nishikawa *et al.*, 1992; Bravo-Angel *et al.*, 1999; Bates *et al.*, 1998).

MobA shows only 18% identity to VirD2 and the VirD2 NLS sequences are poorly conserved, if at all (Bravo-Angel *et al.*, 1999). Despite this, MobA and its cognate *oriT* can substitute for VirD2 and the T-DNA borders to mobilize plasmid DNA (R-DNA) from *Agrobacterium* to plant cells in a *vir*-dependent fashion (Buchanan-Wollaston *et al.*, 1987; Beijersbergen *et al.*, 1992). While comparisons of T-DNA and R-DNA nuclear transfer frequencies are difficult to make due to an inability to measure cytoplasmic transfer frequencies, it is likely that a possible inability of MobA to mediate DNA transfer across the nuclear barrier accounts for no more than a 10-fold decrease in DNA transmission (Bravo-Angel *et al.*, 1999).

Trans-kingdom conjugation experiments with *S. cerevisiae* also testify to a capability of “ordinary’ conjugation proteins to ferry DNA across the nuclear barrier. Indeed, the first reports of trans-kingdom conjugation (bar the “exceptional” *Agrobacterium* story) showed the conjugative transfer of RP4-, F- (Heinemann and Sprague Jr, 1989) and RSF1010-derived (Nishikawa *et al.*, 1992) plasmids from *Escherichia coli* to yeast. Very recently, the *tra* genes of RP4 were shown to mediate conjugative DNA transfer from *E. coli* to cultured mammalian cells (Waters, 2001). The mechanism by which DNA mobilized by conjugation arrives in yeast, plant and even animal cell nuclei has not been reported.

Two models for how DNA from ‘traditional’ conjugative plasmids finds its way to the nucleus of animal cells are conceivable. Either DNA traverses the nucleus by a diffusion-like process, independent of importin-α and the NPC and with or without attached proteins, or the conjugative proteins that are hypothetically attached to the transferred DNA mediate its nuclear import. The second case would be

interesting since it would imply either that conjugative plasmids from bacteria that are not known to have specifically evolved a system for trans-kingdom DNA transfer evolved nuclear localizing ability, or that the nuclear localizing ability inherent within these conjugative proteins is fortuitous.

To test the possibility that the putative conjugative escort proteins TraI and MobA might mediate nuclear transformation via an inherent nuclear localizing ability, fusions of the putative conjugative escort proteins TraI (RP4) and MobA (RSF1010) to EGFP were created. The resulting chimeric proteins were expressed in cultured human cells (INT-407) and their subcellular locations observed by fluorescence microscopy.

MATERIALS AND METHODS

Construction of Fusion Vectors. The bacterial strains and plasmids used in this study are listed in Table 1. Full-length and truncated *traI* and *mobA* variants were amplified from pMWS001 (*traI*) and pMMB207αB (*mobA*) by PCR using a high fidelity *Taq* Polymerase (Expand™, Roche) (see Figs. 1-3 in the Appendix to Chapter 5 for PCR programs and primer design). Primers (Fig. 1 and Table 1, Appendix to Chapter 5) were modified to introduce *Hin* dIII sites upstream of the coding sequences and *Bam* HI sites downstream of the coding sequences. For creation of C-terminal in-frame fusions of the conjugation proteins to EGFP, PCR products were ligated with the PCR cloning vector pGEM-Teasy (Promega) to yield pGCF15a-pGCF18b. *traI and mobA* inserts were liberated from these intermediates by digestion with *Hin* dIII and *Bam* HI and the purified fragments were ligated with *Bam* HI/*Hin* dIII-digested pEGFP-N1(Clontech) to yield pGCF19a-pGCF22b. N-terminal in-frame fusions of the conjugation proteins to EGFP were created directly, by ligating *Bam* HI/*Hin* dIII-digested PCR products with *Bam* HI/*Hin* dIII-digested pEGCF-C1 (Clontech) to yield pGCF23a-pGCF26b. C-terminal in-frame fusions of N-terminal-truncated conjugation proteins to EGFP were created similarly, by ligation of digested PCR products to pEGFP-N1 to create pGCF27a-pGCF28b. Ligated DNA was introduced to freshly competent XL1-Blue by electroporation and transformants were selected on LB agar plates supplemented with kanamycin (Km, 40μg/ml). Plasmid DNA was re-extracted from putative transformants and the ligation of *traI* and *mobA* fragments with EGFP was confirmed by restriction analysis.

TABLE 1. Strains and Plasmids

Strain or plasmid	Relevant genotype and/or phenotype[a]	Source/reference
E. coli strains		
XL1-Blue	*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac*$^-$ F' [*proAB*+ *lacIq lacZDM15 Tn*10] Tc^r	
Plasmids		
pMWS001	Kn^r cassette in *Pst* I site of pMS2260, ColE1 replicon, *traI*+, Cm^r Kn^r	M.W. Silby
pMMB207αB	IncQ, *mobA*+, Cm^r	H.A. Shuman
pEGFP-C1	pUC-based vector encoding EGFP under the human Cytomegalovirus promoter (P_{CMV}) with a multi-cloning site downstream of EGFP. Kn^r/Neo^r	M.A. Kennedy/ Clontech
pEGFP-N1	pUC-based vector encoding EGFP under the human Cytomegalovirus promoter (P_{CMV}) with a multi-cloning site upstream of EGFP. Kn^r/Neo^r	M.A. Kennedy/ Clontech
pGCF19a and b	pEGFP-N1::*traI* (2.195Kb insert). Kn^r/Neo^r. Encodes TraI-EGFP	This study
pGCF20a and b	pEGFP-N1::*traI'* (1.439Kb insert). Kn^r/Neo^r. Encodes TraI'-EGFP	This study
pGCF21a and b	pEGFP-N1::*mobA* (2.126Kb insert). Kn^r/Neo^r. Encodes MobA-EGFP	This study
pGCF22a and b	pEGFP-N1::*mobA'* (0.647Kb insert). Kn^r/Neo^r. Encodes MobA'-EGFP	This study
pGCF23a and b	pEGFP-C1::*traI* (2.195Kb insert). Kn^r/Neo^r. Encodes EGFP-TraI	This study
pGCF24a and b	pEGFP-C1::*traI'* (1.439Kb insert). Kn^r/Neo^r. Encodes EGFP-TraI'	This study
pGCF25a and b	pEGFP-C1::*mobA* (2.126Kb insert). Kn^r/Neo^r. Encodes EGFP-MobA	This study
pGCF26a and b	pEGFP-C1::*mobA'* (0.647Kb insert). Kn^r/Neo^r. Encodes EGFP-MobA'	This study
pGCF27a and b	pEGFP-N1::*traI''* (1.229Kb insert). Kn^r/Neo^r. Encodes TraI''-EGFP	This study
pGCF28a and b	pEGFP-N1::*mobA''* (1.478Kb insert). Kn^r/Neo^r. Encodes MobA''-EGFP	This study

[a] Tc^r, tetracycline resistance; Cm^r, chloramphenicol resistance; Kn^r, kanamycin resistance; Neo^r, neomycin resistance.

Sequencing of Fusion Vectors. The predicted junctions between the inserted fragments and the cloning vector were confirmed by sequencing on an ABI 377XL automated DNA sequencer (Waikato DNA Sequencing Facility, Department of Biological Sciences, The University of Waikato, Hamilton, New Zealand).

Cell Culture. Human intestinal-407 (INT-407) cells (ATCC CCL 6) were cultured in 25cm^2 flasks (Nunc) in Minimal Essential Medium (MEM, Gibco) supplemented with 2mM L-glutamine, 2mM non-essential amino acids (Gibco) and 10% Fetal Bovine Serum (FBS, Gibco) and maintained at 37°C in a 10% CO_2 atmosphere. Cells used in these experiments were between passage 5 and 25.

Transfection. INT-407 cells were harvested from flasks by treatment with 0.25% trypsin, 1mM EDTA (Gibco) and resuspended in cell culture medium to a concentration of 3×10^5 cells/ml and seeded into 24-well trays (0.5ml per well, Grainer) 24 hours prior to transfection. Replicates that were to be fixed, permeabilized and stained with DAPI were seeded onto 13mm glass coverslips placed inside wells.

Plasmid DNA was prepared from *E. coli* XL1-Blue by alkaline lysis using a High Pure Plasmid Isolation kit (Roche). Plasmid DNA concentration was approximated by restriction digestion and gel electrophoresis with visualization of ethidium bromide-stained bands against a marker of known concentration. Cells were transferred to fresh culture medium 30 minutes prior to transfection. Approximately 0.4μg of DNA was introduced to each well along with 1.2μl of FuGene-6 transfection reagent (Roche). FuGene-6/DNA complexes were prepared in serum-free DMEM (Gibco) and introduced to the cell culture medium according to the instructions of the manufacturer (Roche). Transfected cells were incubated for 24 hours before preparation for microscopy.

Microscopy. Cells that were cultured on glass coverslips were fixed and permeabilized with periodate-lysine-paraformaldehyde (PLP) and methanol according to the protocol of Swanson and Isberg (Swanson and Isberg, 1996).

Fixed cells were stained with 0.1μg/ml DAPI for 5 minutes (Swanson and Isberg, 1996), washed 3 times with PBS then mounted on a glass slide with 2.5μl 50% glycerol::PBS and fixed with clear nail varnish.

Cells that were grown on a plastic well surface were dissociated with trypsin. Excess trypsin was removed immediately by aspiration and the cells were incubated at room temperature until detachment was observed. Trypsinized cells were resuspended in PBS supplemented with 1% FBS, pelleted by centrifugation at 1500rpm for 5 minutes and resuspended in a small amount of PBS/FBS (1%). 10μl of cell suspension was pipetted onto a glass slide, overlaid with a glass coverslip and the edges sealed with nail varnish.

Free and fixed cells were screened for EGFP expression at 400x magnification on an Olympus BH2-RFCA epifluorescence microscope fitted with a FITC filter set (O515IF excitation filter, BP495 barrier filter with an EY475 supplementary barrier filter) for visualization of EGFP fluorescence. DAPI-fluorescing nuclei were visualized with a UV filter set (L420 excitation filter, UG-1 barrier filter). Images were captured with a CoolSNAP-Pro™ Digital Camera kit (1392 × 1040 pixels, SciTech) and processed using Adobe Photoshop 6.0 (Adobe Systems). Confocal microscopy was carried out on an Olympus IX70 inverted microscope and a Biorad Radiance confocal scanning system with an argon laser.

RESULTS

Construction of *egfp* fusions to *traI* and *mobA*. Due to its small size and robust fluorescence signal, the Enhanced Green Fluorescent Protein (EGFP) is frequently used as a reporter system to reveal the subcellular locations of proteins of interest. To determine the localization of the putative conjugative escort proteins TraI and MobA when expressed within cultured human cells, EGFP was attached to created both C- and N-terminal in-frame translational fusions, respectively, of *traI* and *mobA.*

Full-length *traI* and *mobA* sequences were amplified from plasmid DNA by PCR and ligated into the multicloning sites of pEGFP-C1 and pEGFP-N1 to create plasmids pGCF19, 21, 23 and 25 (Table 1). Since, in theory, a proportion of PCR products are likely to have incorporated base changes during amplification, duplicate clones of each construct were retained and these are designated ‘a’ and ‘b’. Restriction analysis of the pGCF constructs confirmed that a single copy of each PCR-derived fragment had ligated into the *Hin*d III and *Bam* HI sites of the EGFP cloning vector (Fig. 1). The predicted constructs are depicted in Figure 2.

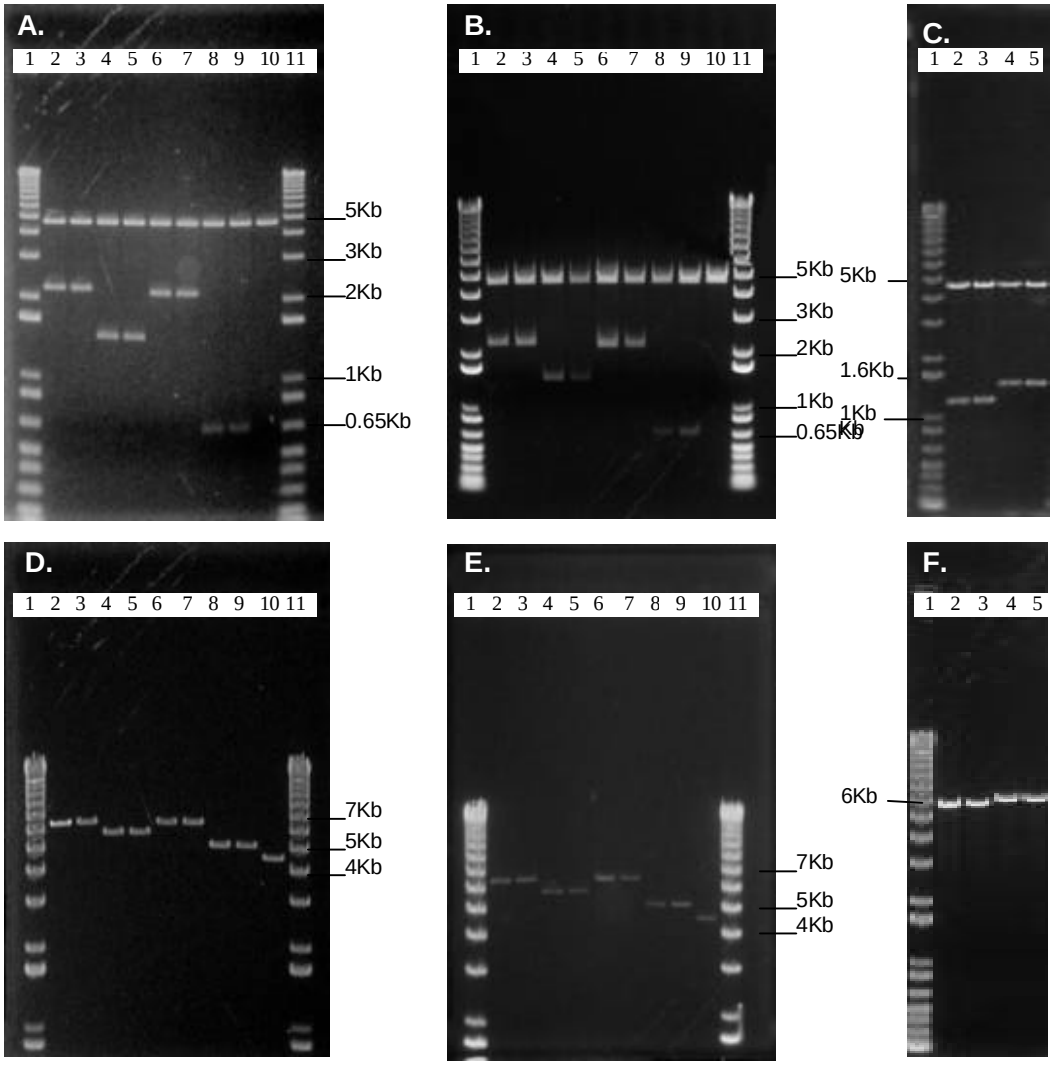

FIG. 1 – Restriction analysis of pGCF19-pGCF28. *Hin*d III/*Bam* HI digests (A) and *Hin*d III digests (D) of pGCF19-pGCF22; lane 2= pGCF19a, lane 3 = pGCF19b, lane 4 = pGCF20a, lane 5 = pGCF20b, lane 6 = pGCF21a, lane 7 = pGCF21b, lane 8 = pGCF22a, lane 9 =pGCF22b, lane 10 = pEGFP-N1.
*Hin*d III/*Bam* HI digests (B) and *Hin*d III digests (E) of pGCF23-pGCF26; lane 2= pGCF23a, lane 3 = pGCF23b, lane 4 = pGCF24a, lane 5 = pGCF24b, lane 6 = pGCF25a, lane 7 = pGCF25b, lane 8 = pGCF26a, lane 9 = pGCF26b, lane 10 = pEGFP-C1.
*Hin*d III/*Bam* HI digests (C) and *Hin*d III digests (F) of pGCF27 and pGCF28; lane 2= pGCF27a, lane 3 = pGCF27b, lane 4 = pGCF28a, lane 5 = pGCF28b. Lanes 1 and 11 = 1Kb Plus DNA ladder (Invitrogen).

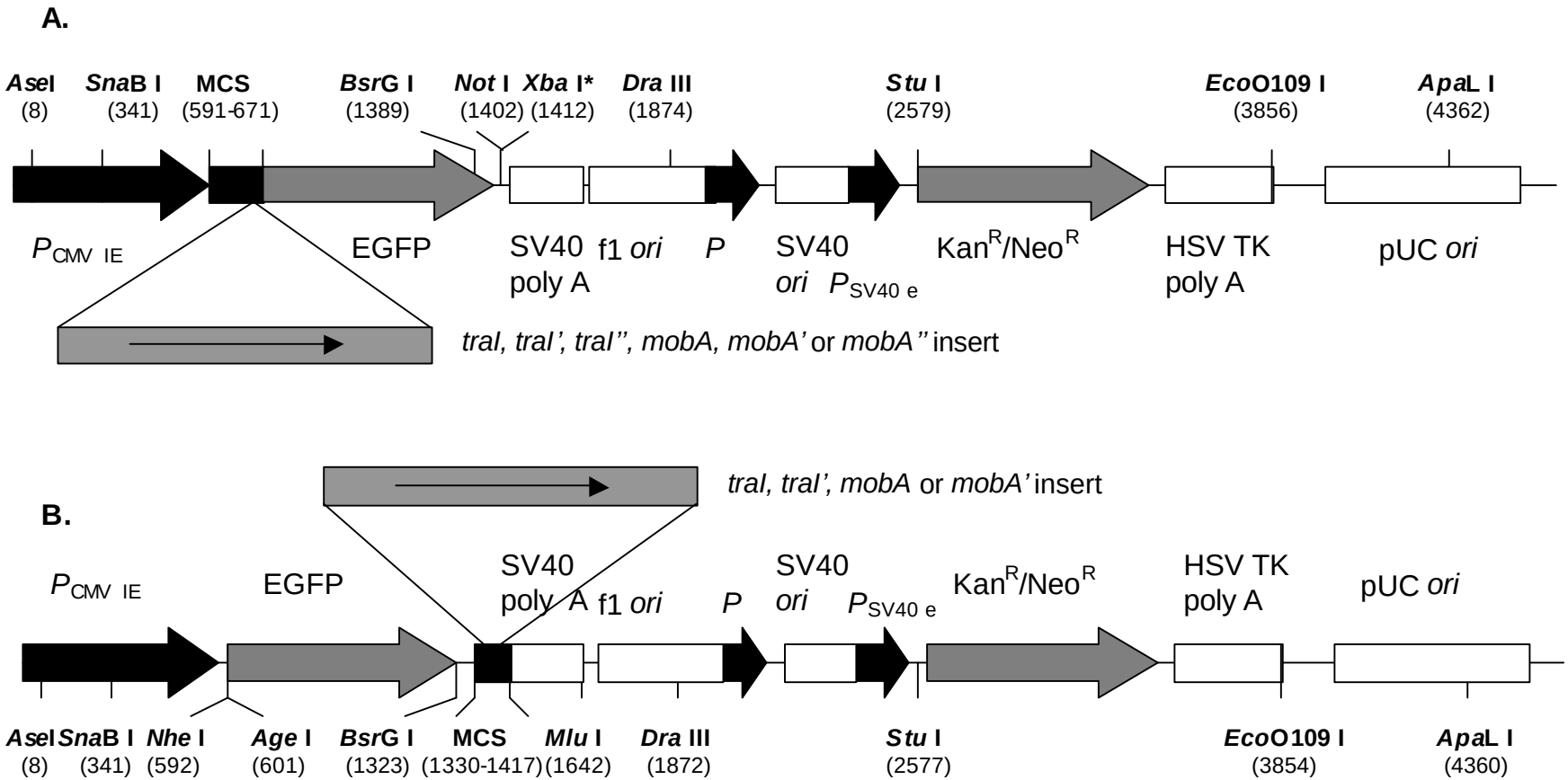

FIG. 2. Maps of pGCF19-28 constructs. Plasmids pGCF19-22 and pGCF27-28 encode C-terminal fusions of TraI and MobA polypeptides to EGFP (A). Plasmids pGCF23-26 encode N-terminal fusions of TraI and MobA polypeptides to EGFP (B).

Confirmation of fusions by sequencing. The junctions between the vectors and the inserted DNA were sequenced on both strands to confirm that precise ligations had occurred. From the sequence data it is expected that the pGCF vectors encode in-frame fusions of TraI and MobA polypeptides to EGFP. The full inserts were not sequenced, but the portion sequenced was found to be identical to the *traI* and *mobA* sequence from Genbank (accession numbers X54459 and NC_001740, respectively) (data not shown).

Expression of fusion proteins in cultured human cells. As expected, EGFP-fluorescence was not observed in *E. coli* carrying the fusion genes because they are driven by the P_{CMV} promoter. That promoter initiates transcription only within animal cells. To test for functional EGFP expression, plasmid DNA was introduced into INT-407 cells by transfection. While some plasmid DNA may integrate into chromosomes and be inherited, the majority of transfectants will be transient, harboring free plasmid DNA that may be expressed but unable to replicate. For the purposes of this study, stable transfectants were unnecessary.

The cells were screened for EGFP expression 24 hours after transfection. Varying intensities of green fluorescence was observed in an estimated 1-10% of cells (with the exception of one experiment where the transfection frequency was estimated at roughly 50%) confirming that 5' insertions of *traI* and *mobA* sequences did not disrupt the reading frame of the *egfp* gene or interfere with functional folding of the EGFP polypeptide. The proportion of fluorescing cells was roughly equal for each plasmid and equivalent to the proportion that fluoresced following transfection with the control plasmids that expressed unmodified EGFP. Thus, there were no obvious differences in the ability of the fusion proteins to be expressed or any evidence of toxicity above and beyond that which might be produced by expression of EGFP alone.

Full-length TraI and MobA fusion proteins localize to the nucleus. Intriguingly, some degree of nuclear localization was observed for both the MobA and TraI

fusion proteins, although considerably more definitively so for TraI (Table 2). TraI-EGFP and EGFP-TraI appeared to localize solely to a subcellular region (Fig. 3) that counterstained with the DNA-binding dye DAPI (Fig. 4). In contrast, MobA-EGFP and EGFP-MobA displayed a range of subcellular locations (Figs. 5, 6 and 7). The MobA fusions could not be said to localize anywhere exclusively. The localization pattern was conserved between permeabilized and unpermeabilized cells (e.g. compare Figs. 3 and 4). Therefore, it is unlikely that differential leaching of EGFP fusion protein from the cytoplasm caused the apparent nuclear localization. No differences in localization were observed between ‘a’ and ‘b’ duplicate vectors for any of the fusion constructs The representative examples of EGFP fusion protein localization within INT-407 transfectants presented herein do not correspond to ‘a’ and ‘b’ plasmids *per se*.

TABLE 2. Nuclear localization of TraI and MobA fusion proteins within cultured human cells

Fusion protein	Degree of nuclear localization
EGFP (pEGFP-C1)	**-**
EGFP (pEGFP-N1)	**-**
TraI-EGFP	**+++**
EGFP-TraI	**+++**
MobA-EGFP	**+/-**
EGFP-MobA	**+/-**

Symbols: -, no nuclear localization; +, weak nuclear localization; ++, moderate to strong nuclear localization with cytoplasmic fluorescence still observed; +++, strong nuclear localization with no cytoplasmic fluorescence observed.

In some cells, MobA-EGFP fluorescence appeared to concentrate in the nucleus (Fig. 6 panels A2 and C1 and Fig. 7. panel A4) whereas in others it appeared to be excluded from the nucleus entirely (Fig. 5 panel 1, Fig. 6. panel A1 and Fig. 7. panels A1 and A3). In others still, no particular subcellular location was observed

(Fig. 5 panels 2 and 3, Fig. 6. panel C2 and Fig. 7. panel A2), with the diffuse distribution of green fluorescence appearing much like that in the EGFP controls (Figs. 8 and 9).

Interestingly, while MobA-EGFP and EGFP-MobA fluorescence was diffused throughout the cell (Fig. 5), TraI-EGFP and EGFP-TraI fluorescence appeared to concentrate predominantly into small subnuclear regions with some diffuse fluorescence visible throughout the nucleus (Figs. 3 and 4). Although in most cases the fluorescent regions were coincident with the DAPI-stained regions of the cell, it was not possible to tell whether these fluorescent structures were located within the nucleus itself or sequestered in the Endoplasmic Reticulum (ER) external to the nuclear membranes.

No difference in the localization pattern was observed between C- and N-terminal EGFP fusion proteins: therefore, it is unlikely that the nuclear location of the fusion proteins was a result of introducing a localization signal at the fusion junctions. Since EGFP alone exhibited no strong subcellular localization pattern (Figs. 8 and 9), it is concluded that localization is conferred by the TraI and MobA regions of the chimeric polypeptides.

Note that ordinary microscopy does not distinguish between true nuclear localization and a mere affinity of the fusion proteins for the external nuclear membranes. Further, ordinary epifluorescence microscopy cannot exclude the possibility that fusion proteins with no apparent nuclear locating ability *do* traverse the nuclear membranes.

Construction of truncated TraI and MobA fusions to EGFP

In the cases of VirD2 and VirE2, inspection of the protein sequence revealed putative bipartite nuclear localization sequences and these were consequently shown to be responsible for the nuclear location of these proteins when expressed

in plant cells (Citovsky *et al.*, 1992; Howard *et al.*, 1992; Tinland *et al.*, 1992; Shurvinton *et al.*, 1992). Similarly, analysis of the TraI (and TraI-EGFP and EGFP-TraI) amino acid sequence by the web-based prediction program PSORT II [http://psort.nibb.ac.jp/form2.html (Bickmore and Sutherland, 2002)] revealed a putative bipartite NLS and a putative monopartite nuclear localization sequence (Fig. 10A). No such candidates were revealed by analysis of the MobA protein sequence (Fig 10B). The TraI sequences aligned reasonably closely with the prototype monopartite and bipartite NLSs and particularly closely with the bipartite sequences from the VirD2 family (Fig. 10C).

A.

```
     1          11         21         31         41         51
     |          |          |          |          |          |
   1 MIAKHVPMRS IKKSDFAELV KYITDEQGKT ERLGHVRVTN CEANTLPAVM AEVMATQHGN  60
  61 TRSEADKTYH LLVSFRAGEK PDAETLRAIE DRICAGLGFA EHQRVSAVHH DTDNLHIHIA 120
 121 INKIHPTRNT IHEPYRAYRA LADLCATLER DYGLERDNHE TRQRVSENRA NDMERHAGVE 180
 181 SLVGWIKREC LPELQAAQSW EDLHRVLREN GLKLRERGNG FIFEAGDGTT VKASTVSRDL 240
 241 SKPKLEARFG AFTPAEGGEA PRRREYRAKP LKTRIDTTEL YARYQSERQE MGAVRKGELD 300
 301 TLRRRRDRLI EAAMRSNRLR RAAIKLLGEG RIAKRLMYAQ AHKALRADLD KINREYRQGR 360
 361 QAVQERTQRR AWADWLKAEA MKGDDKALAA LRAREGRSDL KGNTIQGSGE AKPGHAAVTD 420
 421 NITKKGTIIY RVGSSAVRDD GDRLQVSREA TTDGLDAALR LAMERFGDRI TVNGTAEFKE 480
 481 RIAQAAAAGR LAITFDDAAL ERRRQELLTK EQAHEQPERN DGRRDRGGDG GIRPAAARTT 540
 541 LNATGGDGDR RDARAVSAGG TVALRKPNVG RIGRKPPPQS QNRLRALSQL GVVRIAGGAE 600
 601 MLLPRDVPGH VEQQGAEPAH ALRRGVSGPG RGLKPEQIAA AEKYVAEREQ KRLNGFDIPK 660
 661 HARYTDYVGA LSYAGTRNVE DQALALLRKE NDEILVLPVD KATVQRMKRL AIGDPVTVTP 720
 721 RGSLKTTRGR SR
```

B.

```
     1          11         21         31         41         51
     |          |          |          |          |          |
   1 MAIYHLTAKT GSRSGGQSAR AKADYIQREG KYARDMDEVL HAESGHMPEF VERPADYWDA  60
  61 ADLYERANGR LFKEVEFALP VELTLDQQKA LASEFAQHLT GAERLPYTLA IHAGGGENPH 120
 121 CHLMISERIN DGIERPAAQW FKRYNGKTPE KGGAQKTEAL KPKAWLEQTR EAWADHANRA 180
 181 LERAGHDARI DHRTLEAQGI ERLPGVHLGP NVVEMEGRGI RTDRADVALN IDTANAQIID 240
 241 LQEYREAIDH ERNRQSEEIQ RHQRVSGADR TAGPEHGDTG RRSPAGHEPD PAGQRGAGGG 300
 301 VAESPAPDRG GMGGAGQRVA GGSRRGEQRR AERPERVAGV ALEAMANRDA GFHDAYGGAA 360
 361 DRIVALARPD ATDNRGRLDL AALGGPMKND RTLQAIGRQL KAMGCERFDI GVRDATTGQM 420
 421 MNREWSAAEV LQNTPWLKRM NAQGNDVYIR PAEQERHGLV LVDDLSEFDL DDMKAEGREP 480
 481 ALVVETSPKN YQAWVKVADA AGGELRGQIA RTLASEYDAD PASADSRHYG RLAGFTNRKD 540
 541 KHTTRAGYQP WVLLRESKGK TATAGPALVQ QAGQQIEQAQ RQQEKARRLA SLELPERQLS 600
 601 RHRRTALDEY RSEMAGLVKR FGDDLSKCDF IAAQKLASRG RSAEEIGKAM AEASPALAER 660
 661 KPGHEADYIE RTVSKVMGLP SVQLARAELA RAPAPRQRGM DRGGPDFSM
```

C.

Monopartite:	SV40 large T antigen	p**KKKRK**v
	TraI	p**RRR**ey**R**
Bipartite:	Nucleoplasmin	**KR** paatKKagqa **KKKK**l
	TraI	**RR** dRlieaamRs n**R**l**RR**
	VirD2 (pTiC58)	**KR** pRedddgeps e**RKR**e
	(pTiA6NC)	**KR** pRdRhdgelg g**RKR**a
	(pTiA4b)	**KR** pRveddgeps e**RKR**a

FIG. 10. Protein sequences of TraI (A) and MobA (B). Bold font indicates the putative NLSs (A). The amino acids included in the C-terminal truncated proteins are indicated in red. The amino acids included in the N-terminal truncated proteins are underlined. The putative NLSs of TraI are aligned with the consensus SV40 and Nucleoplasmin signals and also with the bipartite NLS sequences from VirD2 (C). Basic amino acids are indicated in capitals and those from the consensus sequence, in bold.

The necessity of the putative bipartite NLS of TraI for nuclear localization was tested using two TraI deletion constructs. The first, designated TraI', encompassed the first 480 amino acids of the 732aa full-length TraI protein and included both the putative monopartite and bipartite NLS (Fig. 10A). The second, designated TraI'', encompassed the C-terminal 410 amino acids and excluded both putative NLSs (Fig. 10B).

While analysis of the MobA sequence did not reveal any NLS candidates, it was noted that the PSORT II program was an unconvincing predictor of subcellular localization: for example, TraI alone was predicted to accumulate in the nucleus, whereas fusions of TraI to EGFP, at either terminus, were predicted to be cytoplasmic[1]. Alternatively, both MobA fusions to EGFP were predicted to be nuclear whereas MobA alone was predicted to accumulate in the mitochondria. That the range of localization patterns observed with full-length MobA fusions to EGFP included some protein accumulation in the nucleus suggested that perhaps MobA encoded a weak nuclear localizing potential that may be masked by another region of the large fusion protein. For this reason, two sub-peptides of MobA were created. MobA' encodes the first 216 amino acids of the 709aa full-length protein and MobA'' encodes the C-terminal 493 amino acids (Fig. 10B).

TraI and MobA truncated fusion proteins locate in the nucleus. As described previously, pGCF vectors encoding fusions of EGFP and truncated TraI and MobA derivatives, respectively, were constructed (Figs. 1 and 2) and introduced into INT-407 cells by transfection. Fluorescing transfectants were scored for subcellular localization of the EGFP fluorescence after 24 hours (Table 3).

[1] Inconsistencies that arose from the program PSORT II were resolved when an older version of the program, PSORT, was used. The results from both programs are presented in Table 2 within the Appendix to Chapter 5.

TABLE 3. Nuclear localization of TraI and MobA

C- and N-terminal deletion variants

Fusion protein	Degree of nuclear localization
TraI'-EGFP	**+++**
EGFP-TraI'	**+++**
MobA'-EGFP	**++**
EGFP-MobA'	**++**
TraI''-EGFP	**++**
MobA''-EGFP	**-**

Symbols: -, no nuclear affinity; ++, moderate to strong nuclear affinity with cytoplasmic fluorescence still observed; +++, strong nuclear affinity with no cytoplasmic fluorescence observed.

Deletion of the C-terminal region of TraI had no effect on localization of TraI'-EGFP or EGFP-TraI' relative to the full-length fusion protein (Figs. 11 and 12). However, the degree to which the EGFP fluorescence was concentrated into subnuclear regions was perhaps diminished with the TraI' fusion proteins, and the EGFP fluorescence appeared more diffuse throughout the nucleus (compare Figs. 3 and 11). The retention of nuclear localization ability within the TraI' truncated protein is consistent with the hypothesis that nuclear localization is conferred by the putative NLSs within this region. Contrary to this conclusion, however, the N-terminal truncated protein (TraI'') lacking the putative NLS sequences, also accumulated in the nucleus (Figs. 13 and 14). Although the TraI''-EGFP transfectants displayed low level cytoplasmic fluorescence, suggesting perhaps that the deleted NLSs are responsible for some of the nuclear localization activity inherent within TraI, the TraI'' data indicates that the putative NLSs are not necessary for all of TraI's nuclear localization activity.

Of greater surprise was the altered localization pattern of MobA'-EGFP and EGFP-MobA' relative to the full length MobA. Unlike the MobA-EGFP transfectants,

MobA'-EGFP transfectants exhibited a uniform phenotype, with EGFP fluorescence diffused throughout the nucleus (Figs. 15 and 16). In contrast, no particular nuclear affinity of the N-terminal peptide MobA''-EGFP was observed (Figs. 17 and 18), with transfectants appearing much like the EGFP controls (Figs. 8 and 9). Thus, it appears that the first 216 amino acids of MobA may be necessary for the sporadic instances of nuclear localization observed with the MobA-EGFP and EGFP-MobA transfectants (Figs. 7, 8 and 9).

TraI fusion proteins localize to a subnuclear compartment.

Although the TraI fusion proteins appeared coincident with DAPI-stained nuclei, it was possible that the intense concentrations of fluorescence represent protein sequestration in the ER, which is contiguous with the outer nuclear membrane. The apparent subnuclear localization of TraI, TraI' and TraI'' fusion proteins was investigated further using confocal microscopy. Confocal microscopy differs from ordinary epifluorescence microscopy in that fluorescent cells can be visualized as a series of cross-sections. Thus a more detailed breakdown of subcellular fluorescence localization can be generated.

A series of 13 sections were generated for representative TraI-EGFP- and TraI''-EGFP-expressing cells. While fluorescent structures were apparent in each section, including the outermost sections, the fluorescence was strongest in the central region of the cell. Subnuclear fluorescence was coincident with prominent nuclear structures visible in the brightfield images (Figs. 19 and 20). These structures were apparent also in non-fluorescent cells (Fig. 20). Therefore, there structures were not caused by protein aggregates. The subnuclear structures are also visible in the brightfield images taken on the epifluorescence microscope (Figs. 3, 11 and 13). Thus, it appears that TraI localizes to a subnuclear entity although the nature of this entity was unconfirmed by these studies.

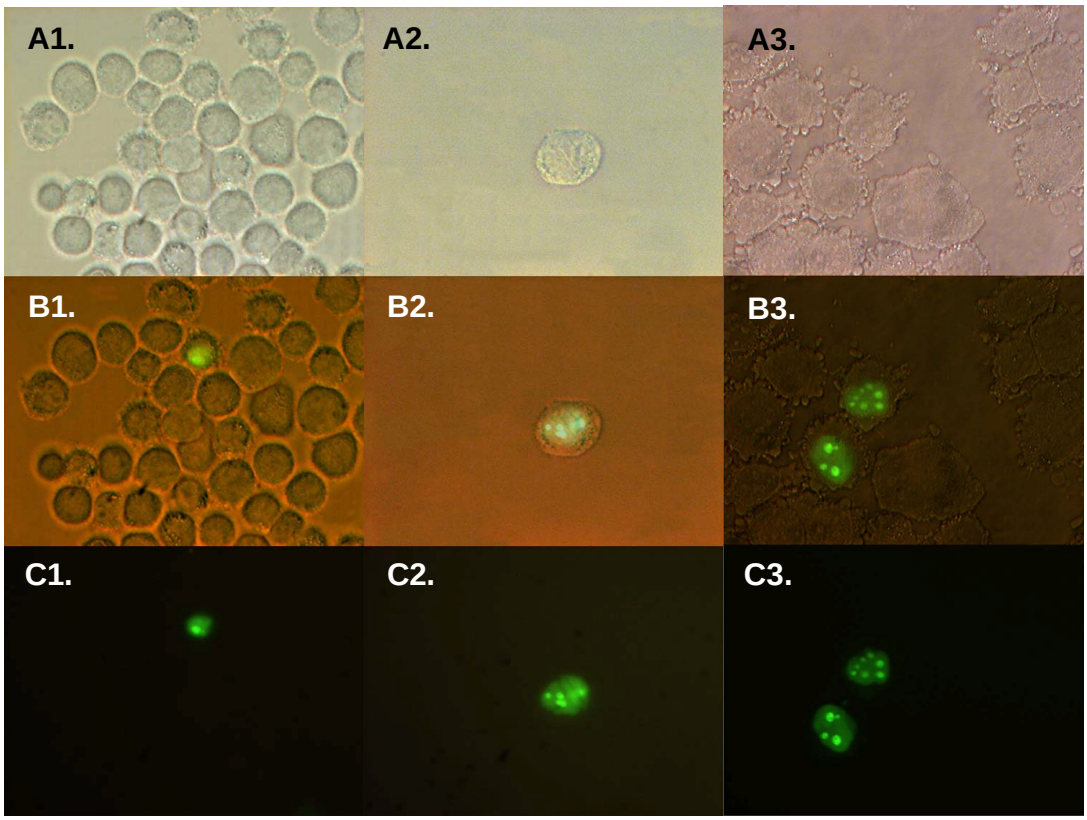

FIG. 3. Location of TraI-EGFP and EGFP-TraI fusions expressed in INT-407 cells. Brightfield images (A) and fluorescence images (C) are overlaid in panel B. Left and centre panels show the location of TraI-EGFP and right panels show the location of EGFP-TraI.

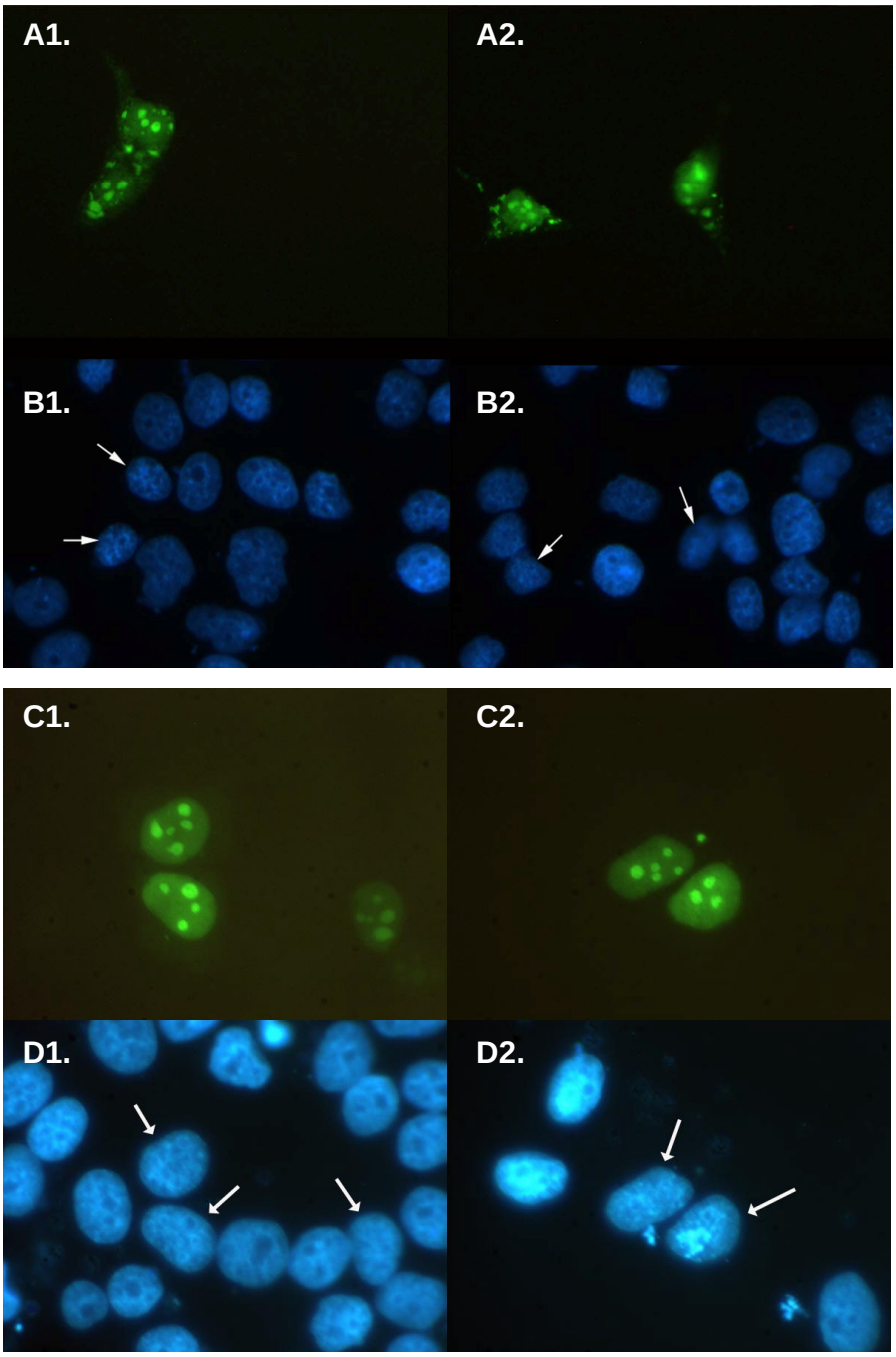

FIG. 4. Co-incidence of DAPI and GFP fluorescence in INT-407 cells expressing TraI-EGFP and EGFP-TraI fusions. Panels A and C show GFP fluorescence. Panels B and D show DAPI fluorescence. Four representative images were selected. Upper panels (A and B) show the location of TraI-EGFP and lower panels (C and D) show the location of EGFP-TraI.

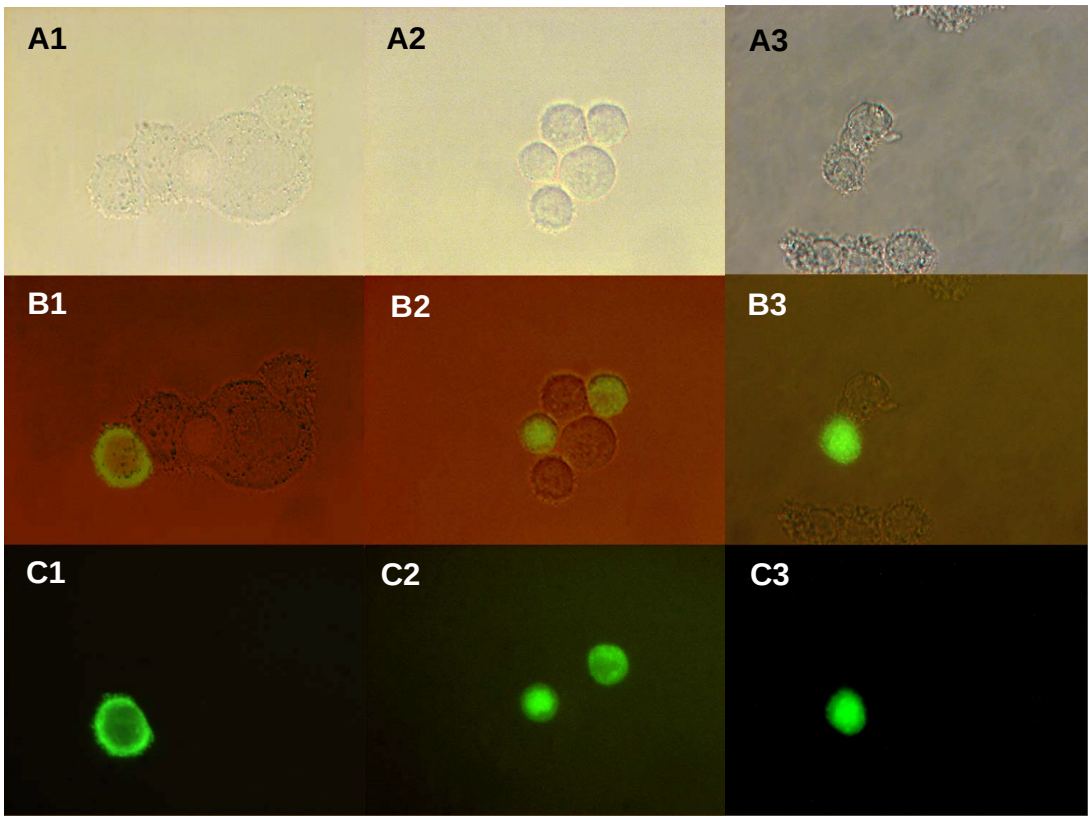

FIG. 5. Location of MobA-EGFP and EGFP-MobA fusions expressed in INT-407 cells. Brightfield images (A) and fluorescence images (C) are overlaid in panel B. Left and centre panels show the location of MobA-EGFP and right panels show the location of EGFP-MobA.

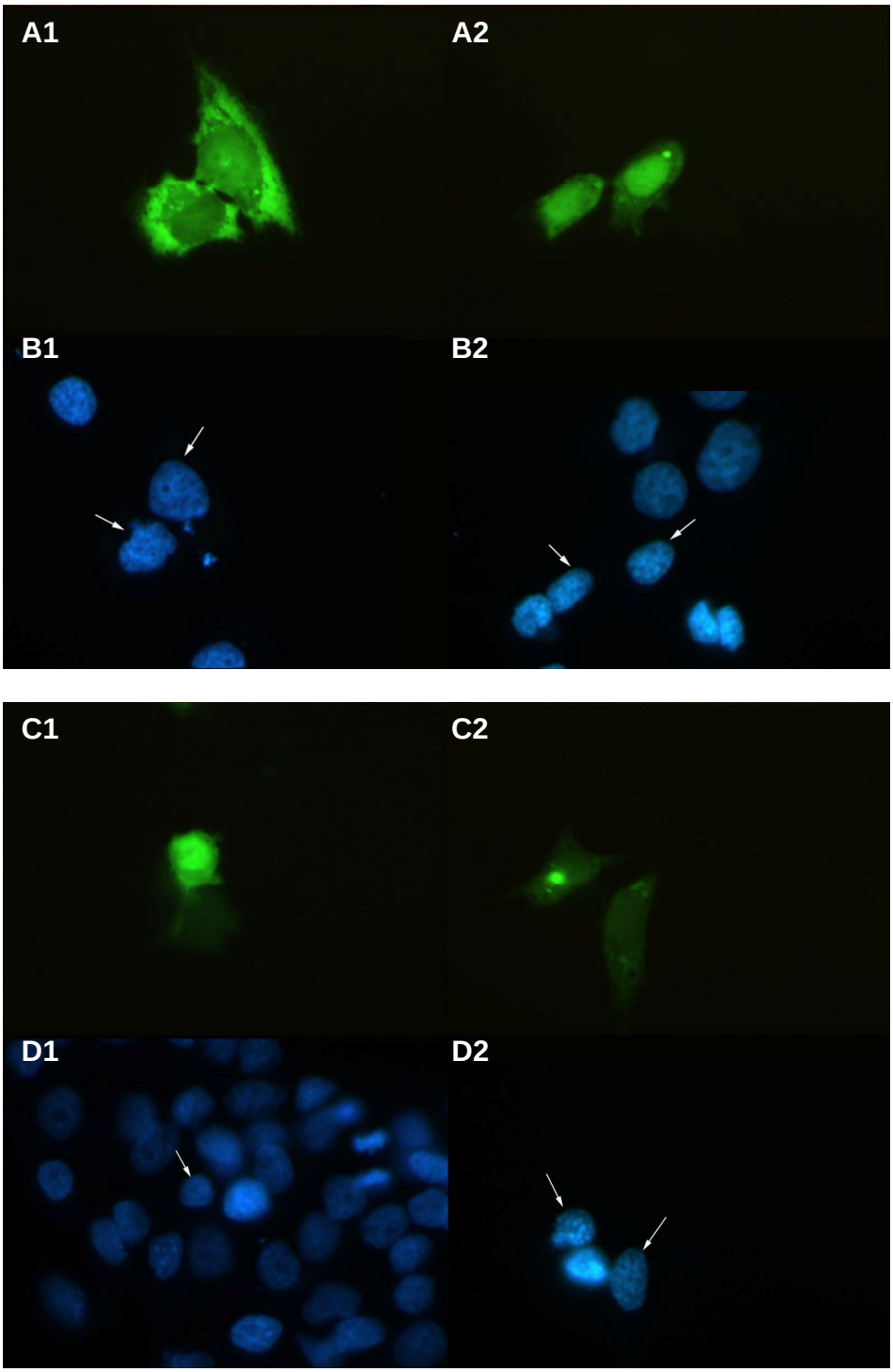

FIG. 6. Co-incidence of DAPI and GFP fluorescence in INT-407 cells expressing MobA-EGFP fusions. Panels A and C show GFP fluorescence. Panels B and D show DAPI fluorescence. Four representative images were selected.

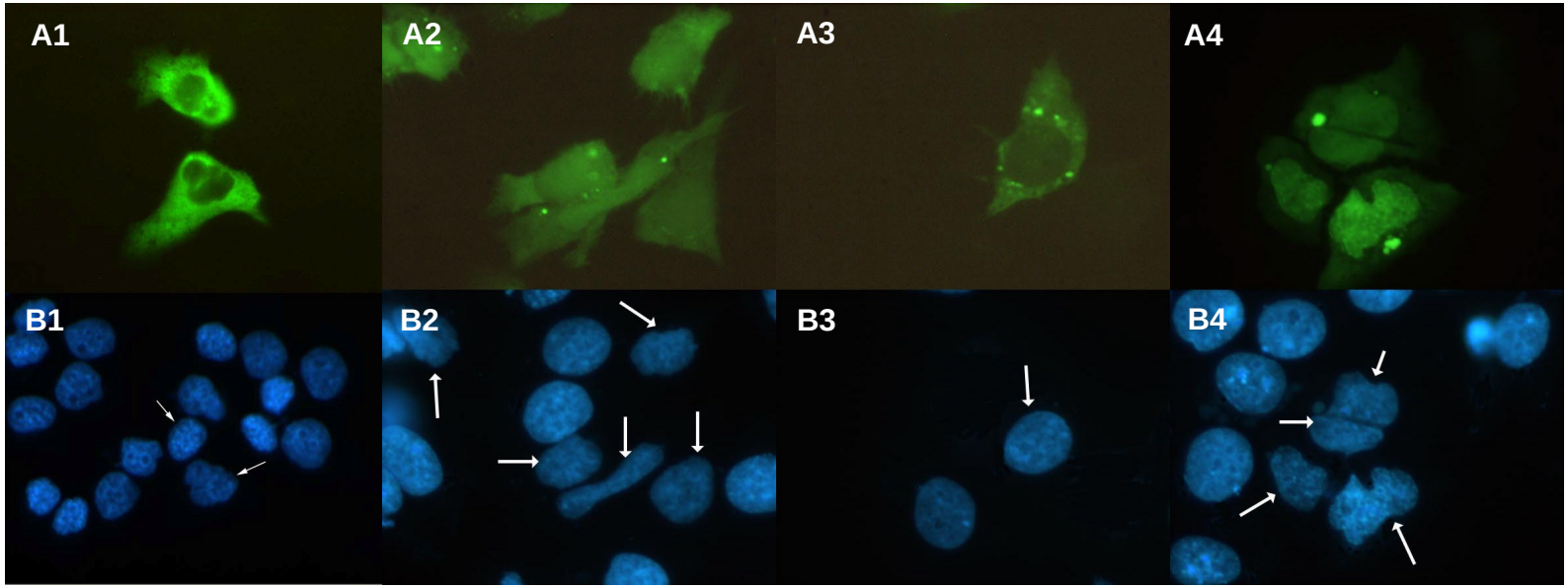

FIG. 7. Co-incidence of DAPI and GFP fluorescence in INT-407 cells expressing EGFP-MobA fusions. Panel A shows GFP fluorescence. Panel B shows DAPI fluorescence. Four representative images were selected.

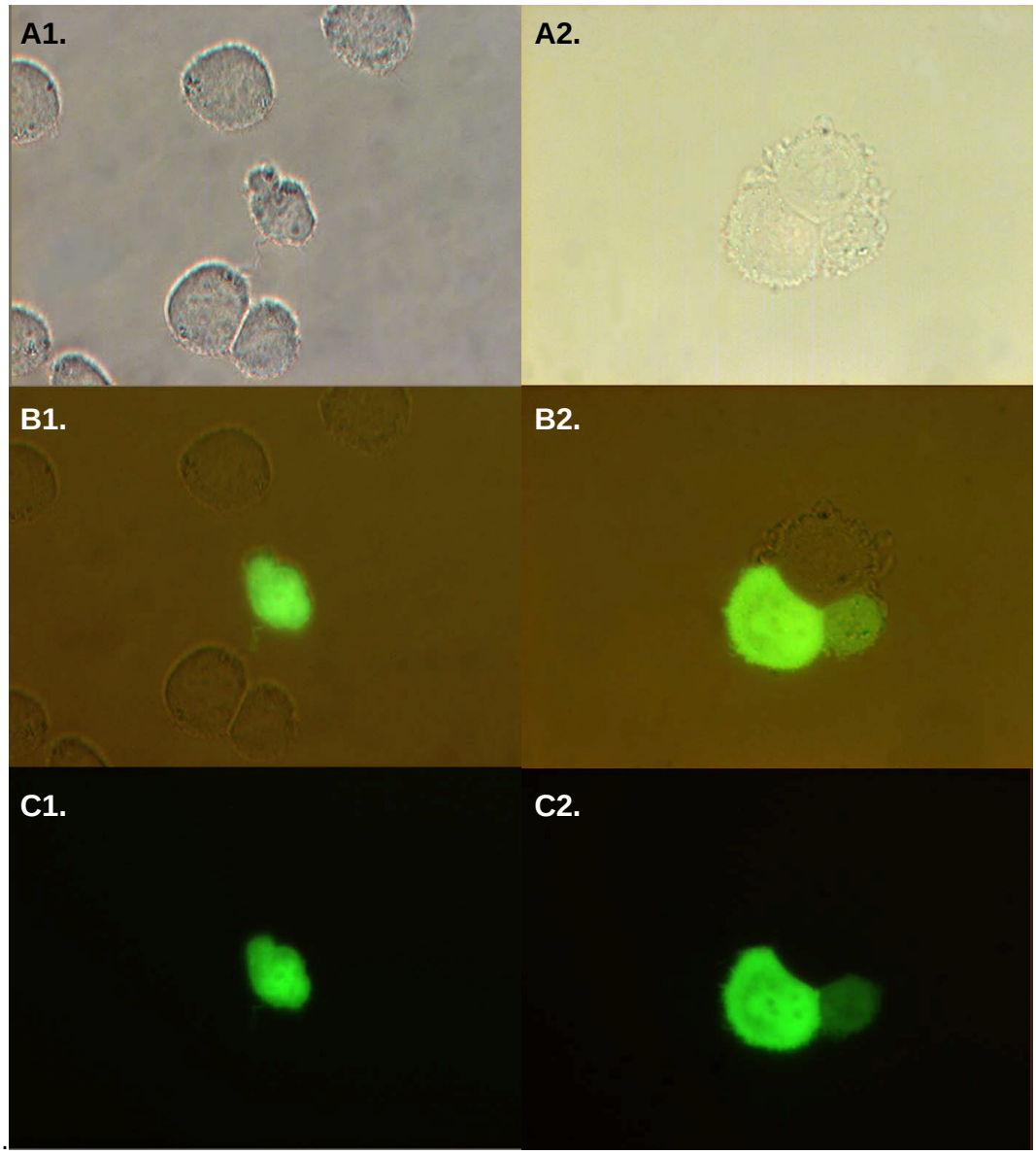

FIG. 8. Location of EGFP expressed in INT-407 cells. Left-hand panels show EGFP expressed from pEGFP-C1. Right-hand panels show EGFP expressed from pEGFP-N1. Brightfield images (A) and fluorescence images (C) are overlaid in panel B.

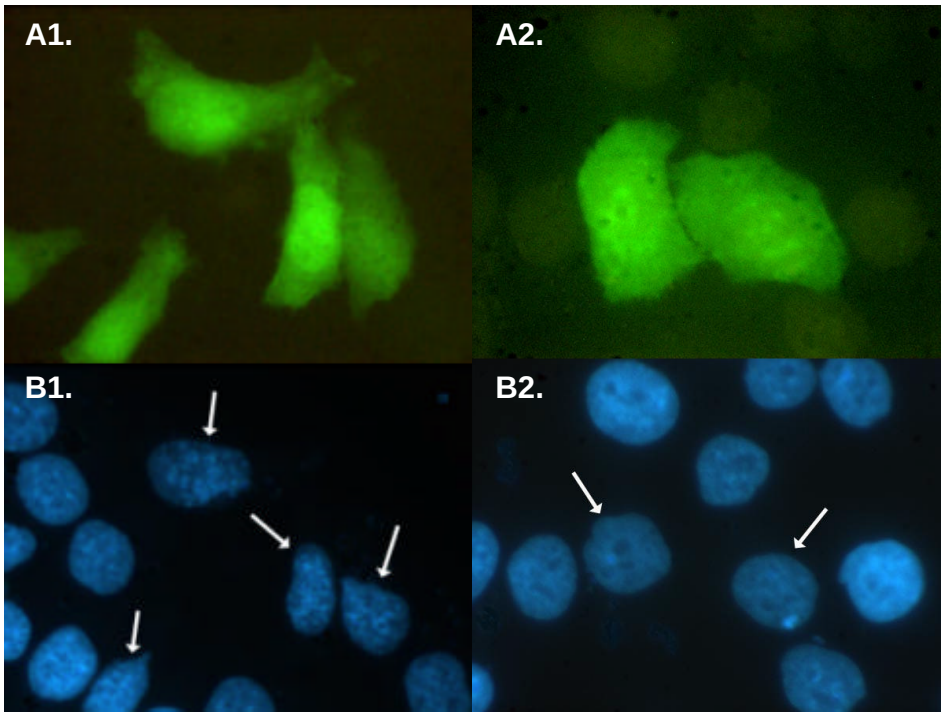

FIG. 9. Co-incidence of DAPI and GFP fluorescence in INT-407 cells expressing EGFP. Panel A shows GFP fluorescence. Panel B shows DAPI fluorescence. Left-hand panels show EGFP expressed from pEGFP-C1 and right-hand panels show EGFP expressed from pEGFP-N1.

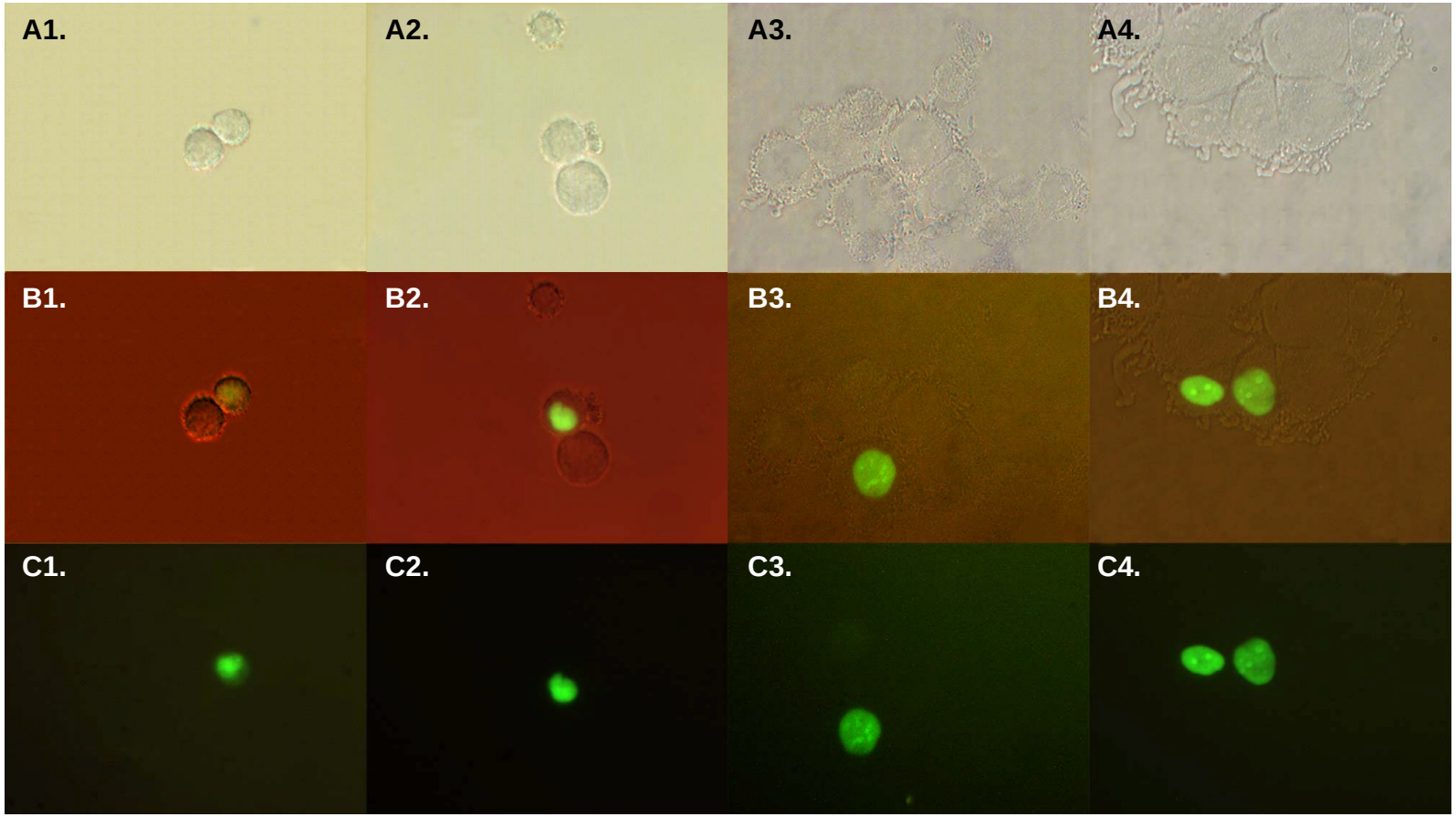

FIG. 11. Location of Tra'-EGFP and EGFP-TraI' fusions expressed in INT-407 cells. Brightfield images (A) and fluorescence images (C) are overlaid in panel B. Panels left of centre show the location of TraI'-EGFP and panels right of centre show the location of EGFP-TraI'.

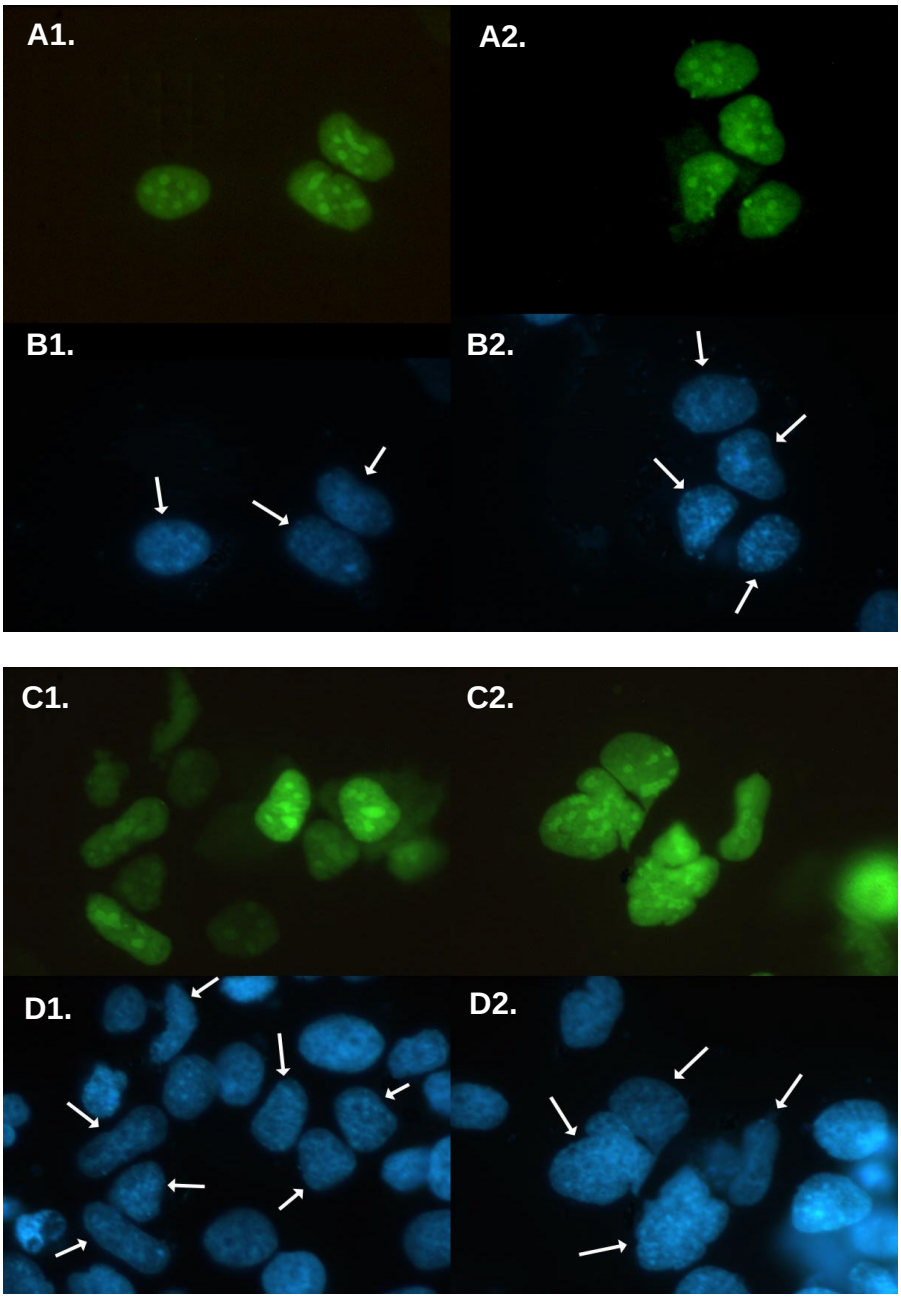

FIG. 12. Co-incidence of DAPI and GFP fluorescence in INT-407 cells expressing TraI'-EGFP and EGFP-TraI' fusions. Panels A and C show GFP fluorescence. Panels B and D show DAPI fluorescence. Four representative images were selected. Upper panels show the location of TraI'-EGFP and lower panels show the location of EGFP-TraI'

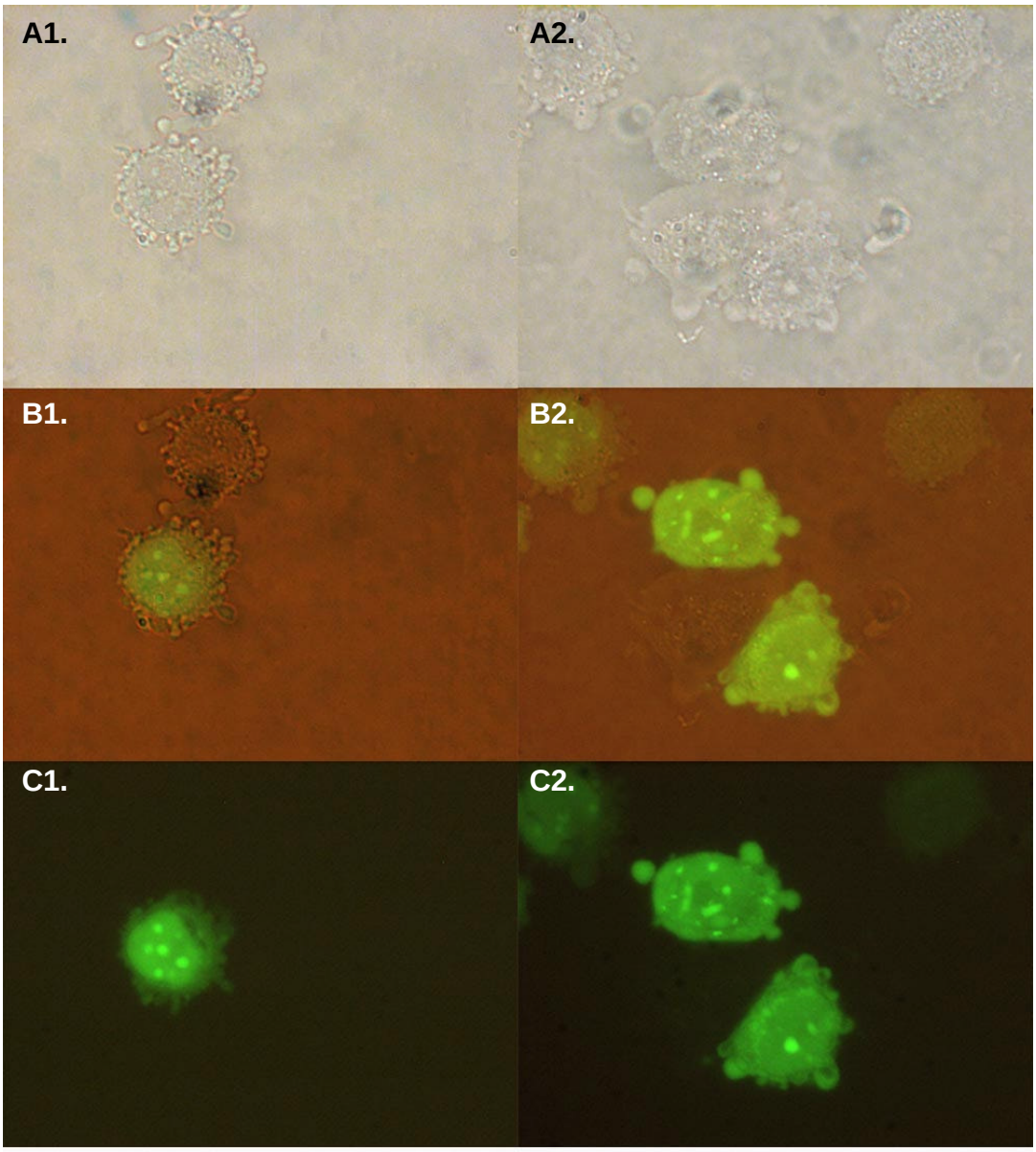

FIG. 13. Location of TraI''-EGFP fusions expressed in INT-407 cells. Brightfield images (A) and fluorescence images (C) are overlaid in panel B. The fluorescent structures on the cell surface are membrane blebs; these occur commonly after treatment with trypsin.

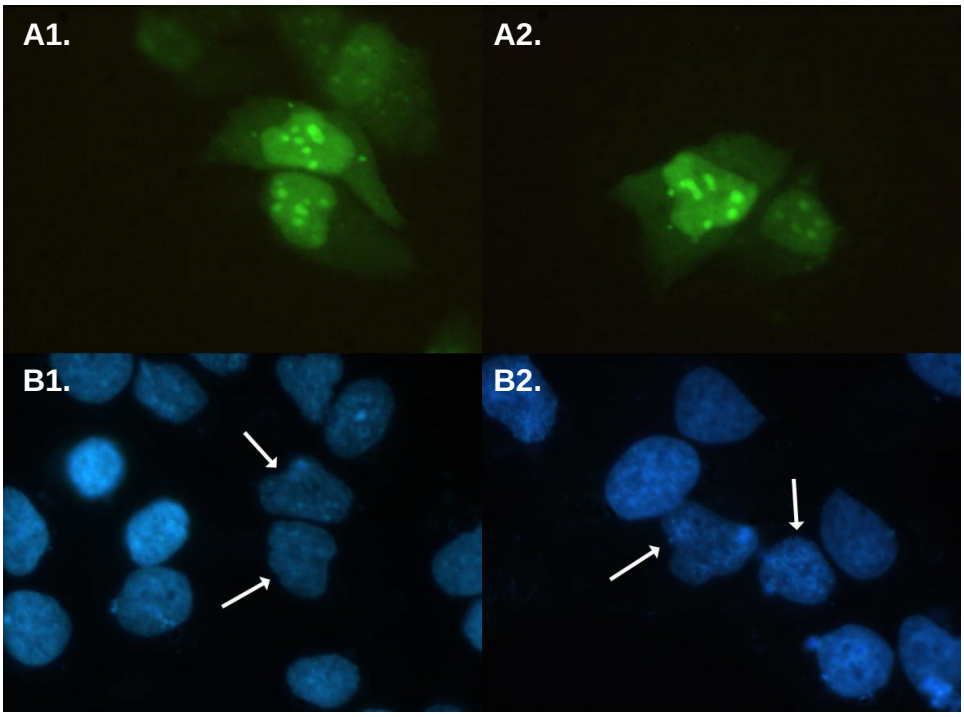

FIG. 14. Co-incidence of DAPI and GFP fluorescence in INT-407 cells expressing TraI''-EGFP fusions. Panel A shows GFP fluorescence and panel B shows DAPI fluorescence. Two representative images were selected.

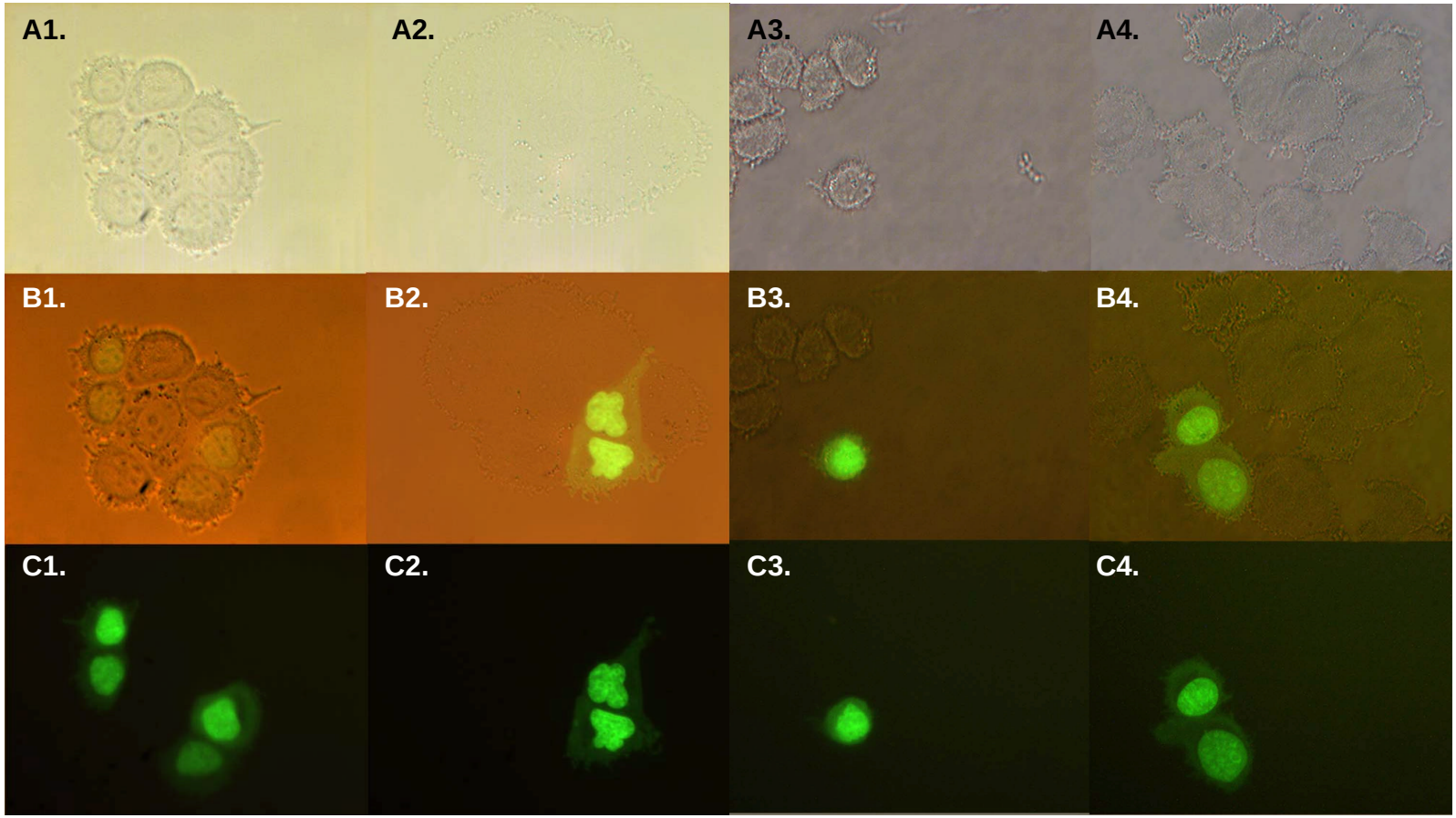

FIG. 15. Location of MobA'-EGFP and EGFP-MobA' fusions expressed in INT-407 cells. Brightfield images (A) and fluorescence images (C) are overlaid in panel B. Panels left of centre show the location of MobA'-EGFP and panels right of centre show the location of EGFP-MobA'.

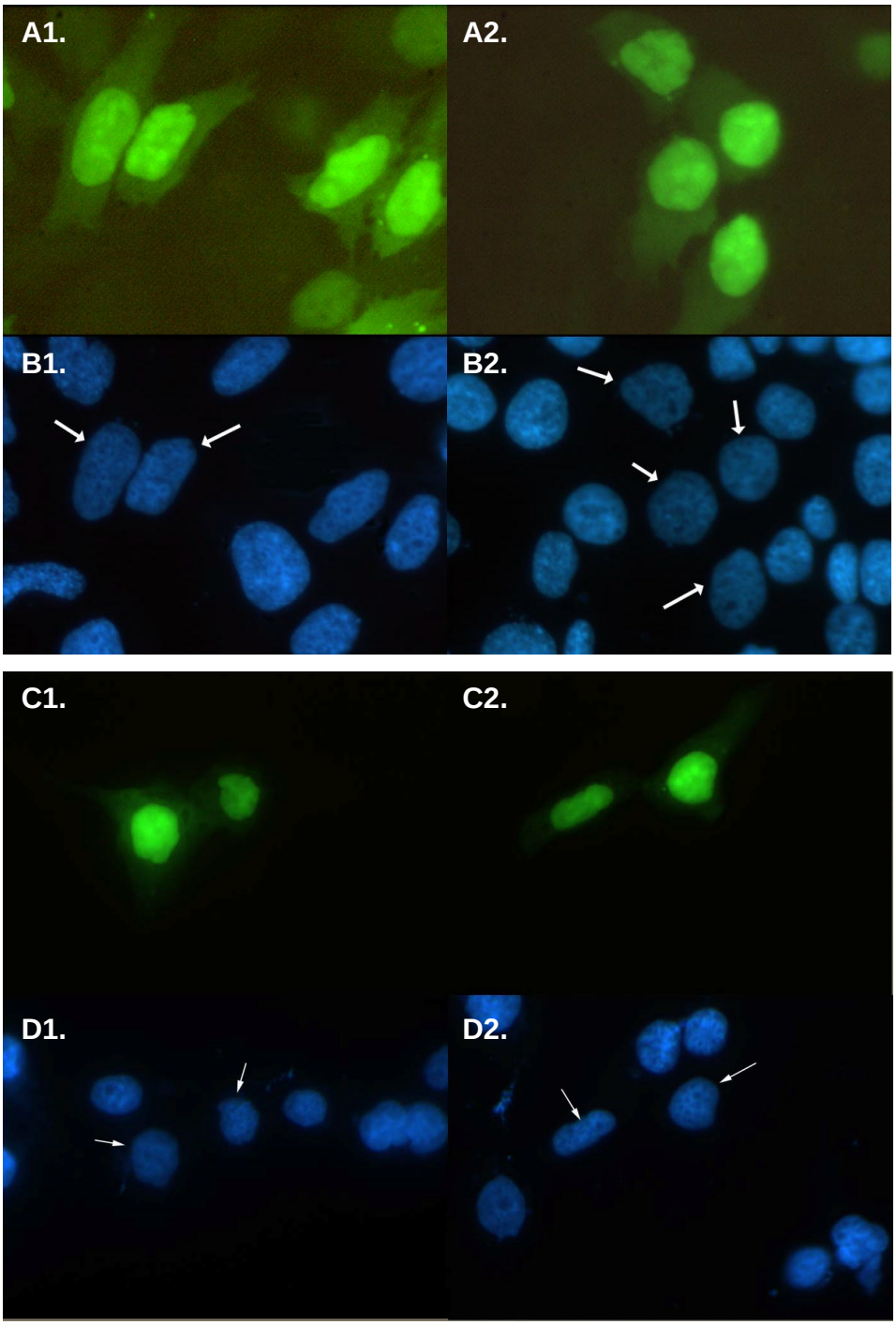

FIG. 16. Co-incidence of DAPI and GFP fluorescence in INT-407 cells expressing MobA'-EGFP and EGFP-MobA' fusions. Panels A and C show GFP fluorescence. Panels B and D show DAPI fluorescence. Four representative images were selected. Upper panels show the location of MobA'-EGFP and lower panels show the location of EGFP-MobA'.

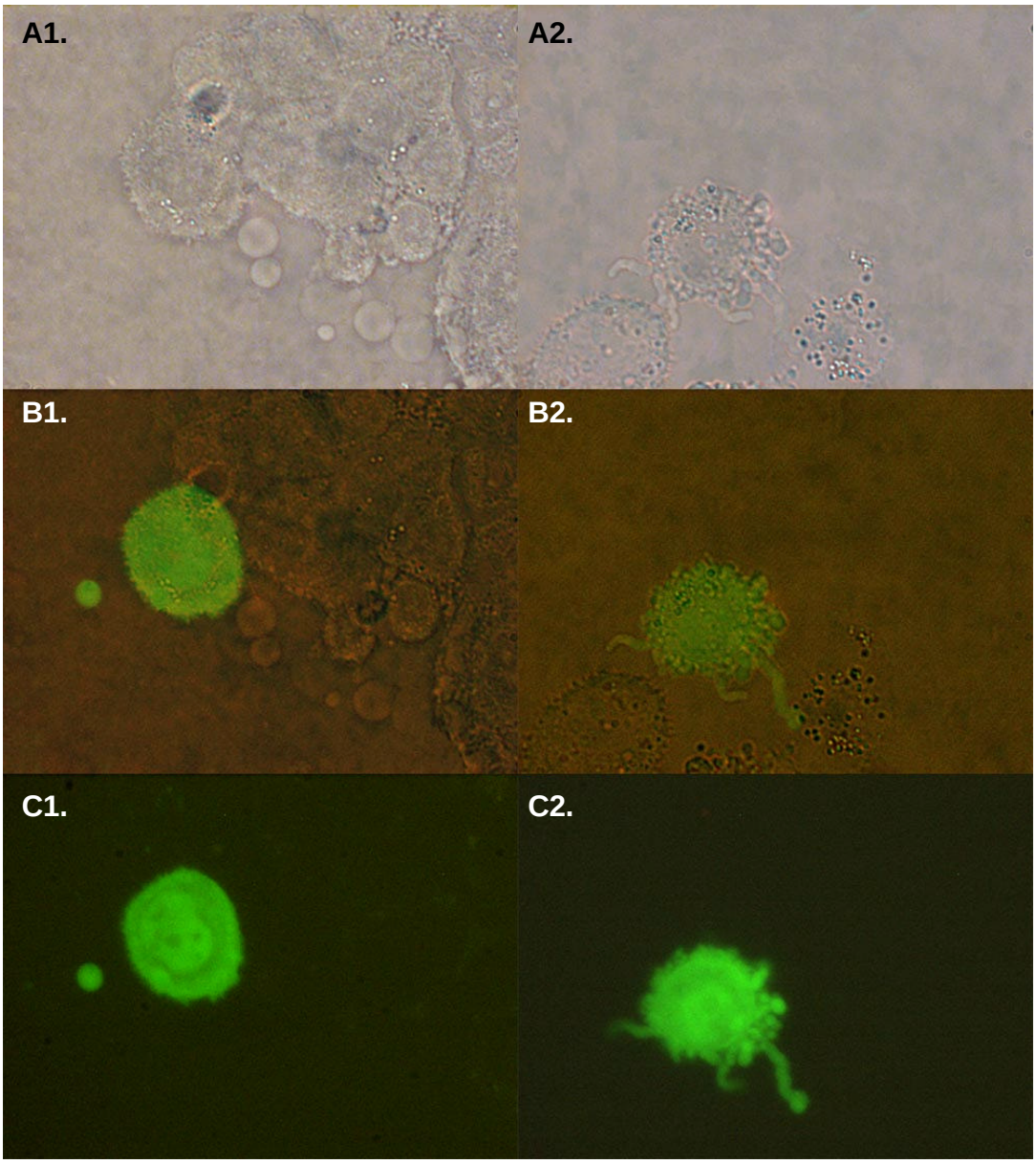

FIG. 17. Location of MobA''-EGFP fusions expressed in INT-407 cells. Brightfield images (A) and fluorescence images (C) are overlaid in panel B. The fluorescent structures on the cell surface are membrane blebs; these occur commonly after treatment with trypsin.

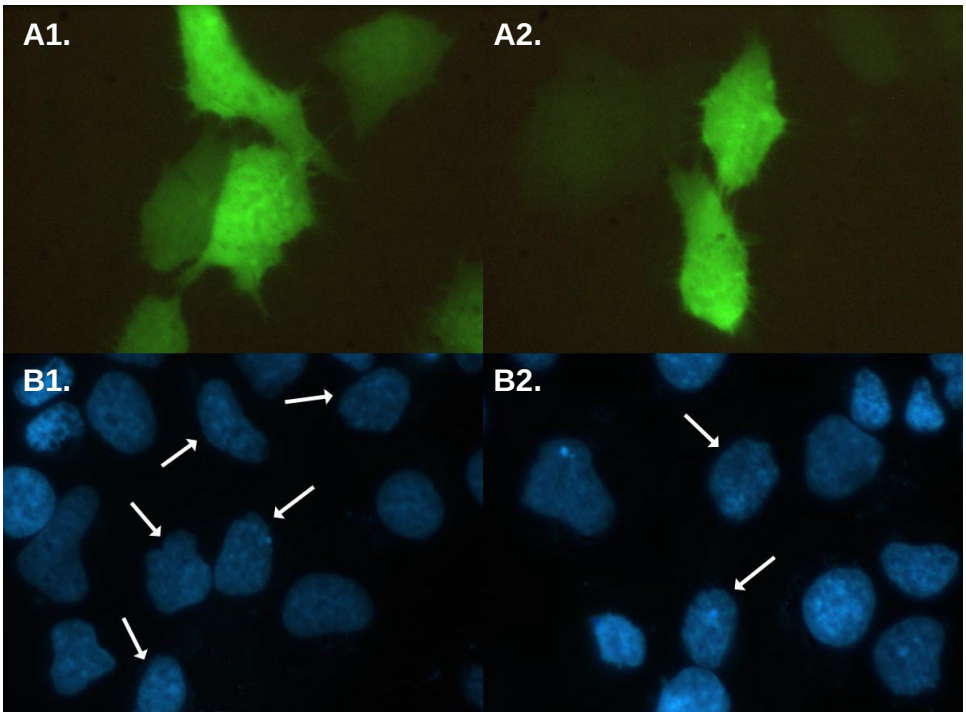

FIG. 18. Co-incidence of DAPI and GFP fluorescence in INT-407 cells expressing MobA''-EGFP fusions. Panel A shows GFP fluorescence and panel B shows DAPI fluorescence. Two representative images were selected.

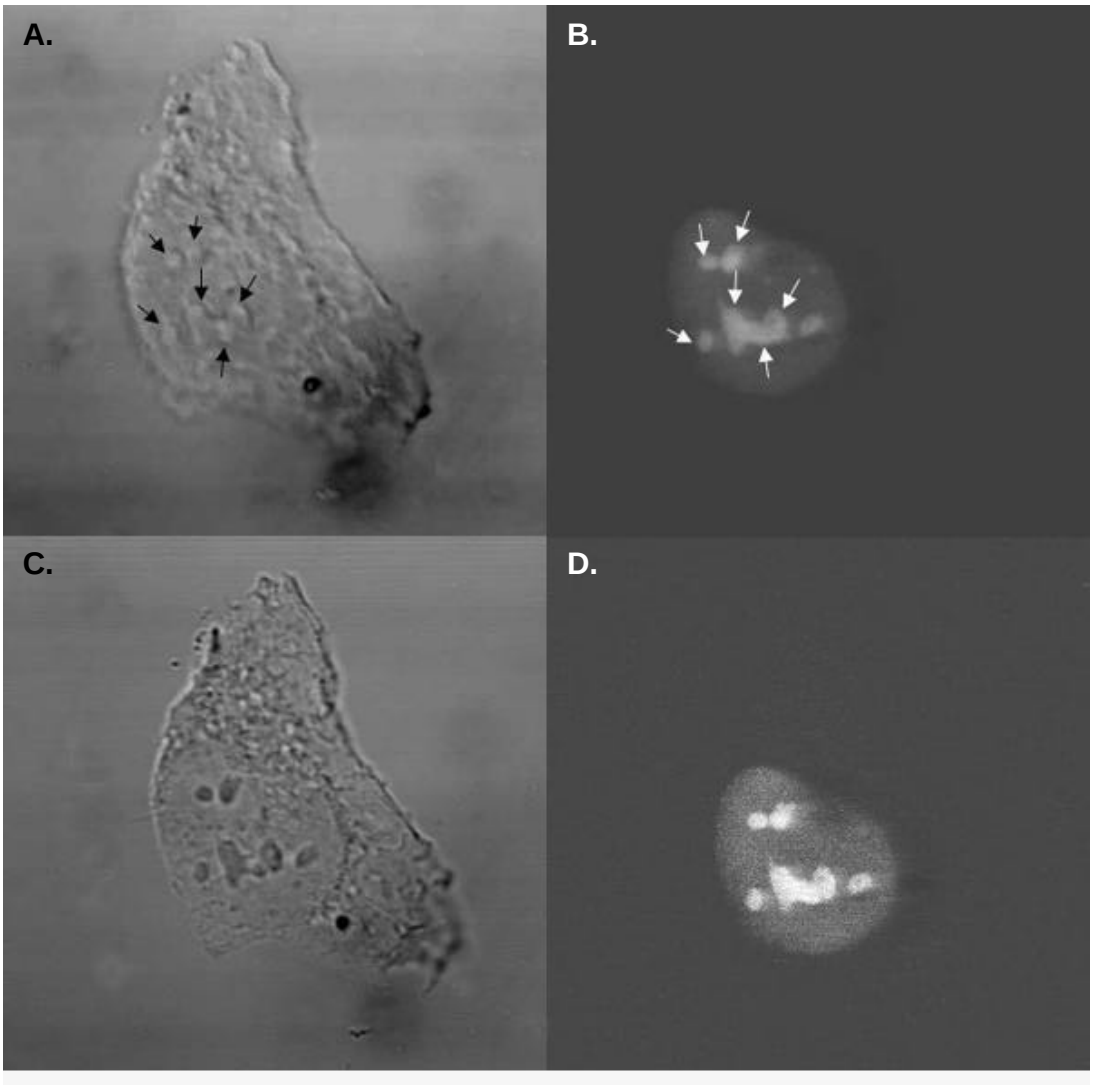

FIG. 19. TraI-EGFP locates within a subnuclear compartment. Panels A and C show brightfield images and panels B and D show GFP fluorescence images. The upper panels represent 13 layered cell sections whereas the lower panels represent one of 13 sections, selected on the basis that it shows most prominently the subnuclear structures in question (indicated with arrows in the upper panels).

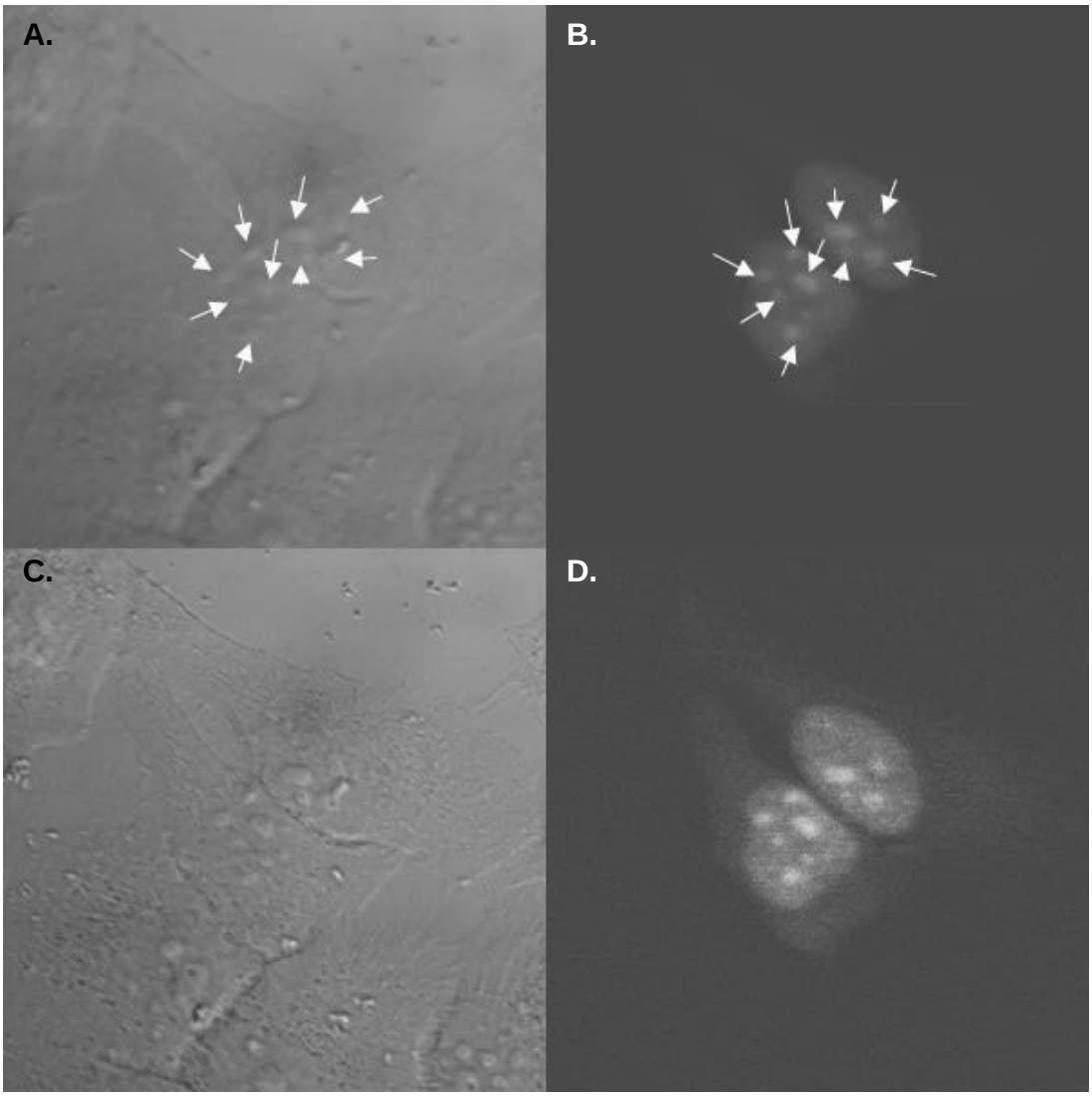

FIG. 20. TraI''-EGFP locates within a subnuclear compartment. Panels A and C show brightfield images and panels B and D show GFP fluorescence images. The upper panels represent 13 layered cell sections whereas the lower panels represent one of 13 sections, selected on the basis that it shows most prominently the subnuclear structures in question (indicated with arrows in the upper panels).

DISCUSSION

When expressed in cultured human cells as fusions to EGFP, TraI its derivatives localized unambiguously and uniformly to the nucleus by an unknown mechanism. Nuclear translocation of proteins above a certain size is believed to occur by an active transport system involving specific protein sequence motifs and interaction with the NPC (Nigg, 1997; Dingwall and Laskey, 1991). Although putative bipartite and monopartite NLSs were identified by at least one predictive program, a TraI deletion variant (TraI'') lacking both these sequences was only partially compromised for nuclear localization. Since both TraI' and TraI'', which represent both non-overlapping 'halves' of TraI, localized to the nucleus, nuclear localization ability must be inherent within *both* halves of the protein. Regions rich in basic amino acids are believed important for nuclear localization. Accordingly, although the C-terminal region of TraI (TraI'') contained no recognizable localization sequences, the proportion of basic amino acids (arginine and lysine) in TraI'' was equally as high as that in the N-terminal region of the protein (TraI') (Table 2, Appendix to Chapter 5). This may serve to explain the similar localization patterns observed with all three TraI derivatives.

Another interesting observation from the TraI localization experiments was the appearance of subnuclear localization. Nuclear TraI fusion proteins and truncated derivatives were observed in bright foci within the nucleus. These fluorescing regions were coincident with prominent subcellular structures that were visible by ordinary light microscopy (Figs. 3, 11, 13, 19 and 20) and were less pronounced in TraI'-EGFP-expressing cells than they were in TraI-EGFP- and TraI''-EGFP-expressing cells (Figs. 11 and 12 vs Figs. 3, 4,13 and 14). Upon closer inspection, some of the fluorescing foci appeared to co-localize with darker regions of the DAPI-stained nuclei, suggesting that TraI and its derivatives localize to regions of the nucleus not containing interphase chromosomes or heterochromatin (e.g. Fig. 4). Subnuclear localization was similarly observed in VirD2 and VirE2 subcellular localization experiments (Relic *et al.*, 1998; Guralnick *et al.*, 1996). In the case of

VirE2, protein (modified to convert the NLS-like sequence to consensus) injected into Xenopus oocytes localized to multiple areas within the nucleus as well as the nuclear periphery (Guralnick *et al.*, 1996). Since VirE2 binds ssDNA and RNA non-specifically, VirE2 binding to DNA within regions of active gene expression most aptly explains the VirE2 subnuclear localization pattern. Such a pattern was not observed for VirD2, which does not bind DNA non-specifically. However, when VirD2 was co-expressed with $VirD2_{\Delta NLS}$-EGFP, green fluorescence within the nucleus was localized to a small number of subnuclear foci that resembled those observed with TraI (Relic *et al.*, 1998). This pattern was similarly observed when VirD2 was co-expressed with VirD1-EGFP. In both cases, nuclear localization of the EGFP fusion proteins is most likely mediated by their binding to VirD2. Thus, the unusual VirD2 nuclear localization patterns were restricted to instances where VirD2 was dimerized with a second protein. Although the subnuclear localization may be explained by properties of VirD1 in the second case, this is unlikely to be the explanation for the $VirD2_{\Delta NLS}$ observation since $VirD2_{\Delta NLS}$ differs from VirD2 only by the loss of two short stretches of amino acids. Thus, the unusual localization patterns in these experiments remain unclarified.

Although the particular subnuclear compartment to which TraI locates cannot be elucidated from the microscopy experiments performed here, on the basis of comparison (for examples see Cáceres *et al.*, 1997; Eilbracht and Schmidt-Zachmann, 2001; Semmes and Jeang, 1996; Catez *et al.*, 2002; Dye and Patton, 2001; Gama-Carvalho *et al.*, 1997; Mao *et al.*, 2002; Sutherland *et al.*, 2001; van Eenennaam *et al.*, 2001; Li and Bingham, 1991) the most likely candidates are either the nucleoli or the cajal bodies (for a review see Spector, 1993). Nucleoli are the sites of ribosome biogenesis and contain the ribosomal genes and their products. Cajal bodies contain ribonucleoproteins (RNPs) and may be involved in the processing, transport and storage of nucleolar metabolites. The cajal bodies are considered nucleolar accessory bodies, being located in close association with (and sometimes indistinguishable from) nucleoli (Leung and Lamond, 2002; Bohmann *et al.*, 1995). Thus, subnuclear localization is best determined by

colocalization studies using antibodies to specific subnuclear proteins such as the nucleolar proteins fibrillin (e.g. Sutherland *et al.*, 2001) or nucleolin (e.g. Catez *et al.*, 2002) and the cajal body protein coilin (Bohmann *et al.*, 1995).

While some more dated studies attempted to determine the specific signal motifs for localization to subnuclear compartments, recent evidence supports a more general mechanism for subcellular localization. For larger proteins, an NLS, or association with a protein containing an NLS, is required for nuclear localization. In contrast, subnuclear localization has largely proven independent of nuclear localization and is most likely mediated by diffusion and retention; retention most likely being determined by more general properties of the nuclear protein (Calado and Carmo-Fonseca, 2000; Hedley *et al.*, 1995; van Eenennaam *et al.*, 2001; Zirwes *et al.*, 1997; Catez *et al.*, 2002). One of these properties, believed to be a strong predictor of subcellular localization, is the isoelectric point (pI) (Bickmore and Sutherland, 2002). pI is determined by the relative proportion of basic and acidic amino acid residues within a protein. Nuclear proteins with lower pIs tend to be located diffusely throughout the nucleoplasm whereas those with higher pIs tend to locate to the nucleoli, cajal bodies and splicing speckles. In agreement with this (although potentially coincidental), TraI has a predicted pI of 9.98 (TraI', 9.62; TraI'', 10.41) and MobA a predicted pI of 6.76 (MobA', 6.01; MobA'', 8.41), consistent with its diffuse nuclear localization (ExPASY http://www.expasy.ch/tools/protparam.html, Table 2 Appendix to Chapter 5). The addition of EGFP to the termini of these polypeptides alters the theoretical pI only marginally (Table 2, Appendix to Chapter 5). Although the identification of common sequence motifs and protein motifs may well be predictive of subcellular and subcellular localization for many proteins, it is important to note that proteins of specific functional families tend to locate together in specific cellular compartments consistent with their function. Therefore, some common motifs may only be indicative of protein function and not a determinant of protein localization. The criteria for subcellular localization of proteins is undoubtedly more complex than is

currently modelled. Experimental systems therefore remain the superior means of determining protein cellular location.

The mechanisms of localization and precise location of TraI within the nucleus remain undescribed. Although a nucleolar location for nuclear TraI is an attractive hypothesis, it cannot be ruled out that the apparent subnuclear localization is artifactual, caused by either aggregation of over-expressed protein (Hedley *et al.*, 1995) or an effect of protein expression in a heterogeneous background. For these reasons it would be prudent to confirm the localization observations, for both TraI and MobA, by complementary methods. For example, microinjection of fluorocin-tagged native proteins into the highly nucleated cells of Xenopus oocytes proved a successful technique for demonstrating the subcellular affinities of VirD2 and VirE2 (Guralnick *et al.*, 1996).

Determination of the precise mechanisms of TraI nuclear and subnuclear localization is beyond the scope of this study. However, two theoretical models are proposed. In the first, TraI nuclear localization is mediated by sequence motifs/properties encoded within the protein itself, these not necessarily conformable to the prototypic consensus motifs. Nuclear localization is not always predictable from a protein's sequence. In support of this, only 62% of cajal body proteins and 41% of nuclear periphery proteins have a recognizable NLS (Bickmore and Sutherland, 2002). In the second model, TraI nuclear localization is mediated by association of TraI with a second protein, the second protein harbouring the nuclear localization potential. This is exemplified by VirE2, which localizes to plant cell nuclei by association with VIP-1 (Tzfira *et al.*, 2001). Following nuclear uptake, TraI proteins may diffuse into a subnuclear compartment and be retained there by virtue of their basic charge.
Both of these models require that either protein-association or nuclear-import domains be present independently within *both* TraI' and TraI''.

The case of MobA is more difficult to interpret. Full-length fusions of MobA to EGFP showed cytoplasmic localization with nuclear exclusion in a predominance of cells (Figs. 5,6 and 7). On the basis of this observation, it could be argued that MobA contains no inherent nuclear localization ability. There was, however, a proportion of transfectants that showed either nuclear accumulation of MobA or a diffuse cellular location with no apparent exclusion from the nucleus. One interesting possibility is that entry of MobA-EGFP to the nucleus is cell cycle-dependent. Proteins may enter the nucleus during cell division, when the nuclear envelope has temporarily disassembled, and be retained there (Sutherland *et al.*, 2001). The possibility of cell cycle-dependent MobA nuclear entry was proposed by Bravo-Angel *et al.*, who found unusual patterns of integration of T-DNA into plant chromosomes when transfer was mediated by MobA (Bravo-Angel *et al.*, 1999). Paradoxically, in spite of a low frequency of MobA-mediated nuclear transformation relative to T-DNA transferred by VirD2, when rare recombinant plants were regenerated their cells were found to contain multiple integrated copies of the T-DNA with many copies found to be nested within another. It was suggested that multiple copies of MobA-T-DNA complexes accumulated cytoplasmically and entered the nucleus simultaneously at prophase, when the nuclear membranes were disassembled during mitosis.

A complicating observation is the nuclear localization of MobA'-EGFP (Figs. 15 and 16). MobA' fusion proteins accumulated strongly and diffusely within the nucleus. Thus it would appear that deletion of the C-terminus of MobA alters the protein in some way that facilitates its nuclear entry. A further complicating observation was the apparent lack of exclusion of MobA''-EGFP from the nucleus (Figs. 17 and 18). While MobA-EGFP-expressing transfectants exhibited dark regions corresponding to the nucleus, MobA''-EGFP fluorescence appeared diffuse throughout the cell. Proteins greater than 45kDa are generally regarded as nuclearly-excluded. However, a fusion protein of 65kDa was reported to enter the nucleus by passive diffusion (Jagiello *et al.*, 2000). The predicted (ExPASy) size of MobA''-EGFP is 83kDa, considerably larger than both the 45kDa exclusion limit and the 65kDa

exception. An alternate possibility is passive uptake of MobA''-EGFP via association with a nuclear-localizing protein. EGFP alone is only 28kDa and *does* traverse the nucleus by passive diffusion (Figs. 8 and 9) (Jagiello *et al.*, 2000). At 52kDa, MobA'-EGFP may also be capable of passive nuclear entry.

From these observations, three predictive models for MobA' nuclear localization can be envisaged. In the first, MobA enters the nucleus in a cell cycle-dependent fashion (or in the case of MobA'-EGFP, by passive diffusion). In accordance with this, MobA must simultaneously be retained in the nucleus, possibly by a specific mechanism. The failure of MobA''-EGFP to be retained in the nucleus thus predicts that the nuclear retaining ability resides within the N-terminal 216 amino acids of MobA. The model makes the further prediction that should the cell cycle be synchronized, a uniform localization pattern would be observed in cells expressing MobA-EGFP.

In the second model, MobA' localizes to the nucleus by way of a cryptic NLS. This model predicts firstly that the NLS be present in the N-terminal 216 amino acids since MobA''-EGFP did not localize to the nucleus. Secondly, model two predicts that the nuclear localization be masked, to a certain extent, within the full-length MobA protein.

The final model proposes that MobA' nuclear localization occurs via association with another protein that carries nuclear localizing potential. This model predicts that the association occurs via the N-terminus of MobA and is only formed (or readily formed) in the absence of the C-terminus.

How do these findings relate to trans-kingdom conjugation? To date, transfer of TraI, MobA and other conjugative proteins into recipient cells as protein-DNA complexes has not been demonstrated. Nor has such transfer been formally demonstrated for VirD2-T-DNA complexes in *A. tumefaciens*. However, a role for conjugative proteins in T-DNA transfer, nuclear transformation and integration in

the *Agrobacteria* system is strongly supported. A model for the involvement of proteins as DNA chaperones is indeed compelling, especially in light of the recently unveiled homology between protein- and DNA-secretion systems. The results presented here suggest that the VirD2 homologue TraI (Pansegrau *et al.*, 1993b) too encodes the potential to mediate nuclear transformation. This potential, however, awaits rigorous test.

Ideally, any test of the necessity of nuclear localization potential for TraI-mediated trans-kingdom conjugation requires a TraI mutant lacking nuclear localization domains but retaining its DNA processing functions. Unfortunately, the region of the TraI polypeptide responsible for nuclear localization was not located in this study and appears to be more complex than the case of VirD2 where nuclear localization can be fully attributed to either one or both identified consensus signals (Relic *et al.*, 1998; Shurvinton *et al.*, 1992; Tinland *et al.*, 1992; Howard *et al.*, 1992). An alternative assay might make use of the non-hydrolysable GTP analog, GTPγS, which blocks Ran/TC4 GTPase and inhibits the nuclear import pathway. Although, such a test would depend upon the untested assumption that TraI nuclear translocation occurs via a traditional nuclear import pathway.

Although translocation of full-length MobA fusion proteins to the nucleus was not regularly observed, nuclear MobA *was* observed in some transfected cells. As discussed previously, MobA may traverse the nuclear membranes at some low frequency by virtue of its native properties. The alternate possibility is that MobA gains access to the nucleus in a cell cycle-dependent fashion (Bravo-Angel *et al.*, 1999). If the latter were the predominant mechanism of MobA nuclear entry (and the explanation for the observed MobA-EGFP nuclear localization in a proportion of transfected cells observed here), then it is likely that other conjugatively-mobilized DNA molecules and/or protein-DNA complexes too enter the nucleus in this non-specific fashion. This would abrogate the value of an NLS within the co-transferred proteins at least to some extent. Certainly, if lacking in a refined mechanism of nuclear entry, this does not appear to dramatically effect the ability of MobA to

mediate trans-kingdom conjugation because RSF1010 transmits to plant and yeast cells (Bates *et al.*, 1998; Bravo-Angel *et al.*, 1999; Nishikawa *et al.*, 1992) at high frequencies relative to that of IncP plasmids and T-DNA (Bundock *et al.*, 1995; Heinemann and Sprague Jr, 1989; Nishikawa *et al.*, 1990).

When the efficiency of MobA-and VirD2-mediated T-DNA nuclear transfer and transmission from *Agrobacteria* to plant cells was compared, MobA-mediated nuclear transfer was found to be two orders of magnitude less efficient than VirD2-mediated nuclear transfer (Bravo-Angel *et al.*, 1999). However, at least one order of magnitude difference was accounted for by the inhibitory effect of MobA on VirE2 translocation into plant cells. As the MobA-contributing plasmid was itself mobilizable, and MobA known to compete with other translocated substrates for the VirB translocation pore (Stahl *et al.*, 1998), it was not certain what proportion of the remaining deficiency in MobA-mediated nuclear transfer could be attributed to an inability of MobA to traverse the nuclear membranes. At the most, it would appear that a possible nuclear barrier would account for only a 10-fold decrease in MobA-mediated T-DNA transfer (Bravo-Angel *et al.*, 1999). A cell cycle-dependent model for MobA-mediated trans-kingdom gene transmission is indeed plausible and makes a testable prediction: that the gene transmission frequency should be increased when recipient cells are actively dividing and decreased when recipient cells are stationary. This should be most readily testable using the *E. coli* x yeast experimental system.

Although nuclear translocation may indeed be a barrier to trans-kingdom gene transmission, Bates *et al.* suggest that the major barrier is in fact the ability of the putative 'mating pore' to mediate transfer from the donor bacterium to the recipient cell (Bates *et al.*, 1998). While IncPα 'mating pore formation' (mpf) genes were able to mediate transfer of both IncPα- and IncQ-derived shuttle vectors (encoding *oriT*s and cognate mobilization functions) to yeast recipients, IncI1 and IncF1 mpf systems were unable to mediate transmission of either themselves or the shuttle vectors to yeast at a detectable frequency, suggesting an inherent supremacy in

the ability of the IncPα mating apparatus to form permissive associations with yeast recipient cells. Although the IncPα and IncQ conjugation frequencies generated in this study are not *directly* comparable (since they were not performed in parallel) the short duration of the conjugation experiments (one hour) makes the frequencies *approximately* comparable. Mobilization of the MobA-shuttle plasmid by the IncPα mpf genes thus occurred at a frequency ~20-fold below that of the TraI-shuttle plasmid. In comparison, mobilization of these plasmids to *E. coli* occurred at equal frequencies. The significance of the 20-fold decrease is uncertain. Since IncQ yeast transconjugants were readily obtainable, it would appear that efficient nuclear transformation by MobA is not highly essential for trans-kingdom conjugation.

Thus, to conclude tentatively, it is possible that absence of nuclear localization potential within putative conjugative escort proteins may not be a major barrier to their mediating trans-kingdom conjugation. However, more sensitive genetic experiments are necessary to determine MobA's true nuclear localizing potential. In an example of such, Vergunst *et al.* used the Cre/*lox* system to demonstrate translocation of VirE2 and VirF into plant cells from *A. tumefaciens*. A disrupted, *lox* excision site-flanked, drug resistance marker was inserted into the plant genome and the frequency of drug resistance restoration scored following mating with *A. tumefaciens* strains expressing fusions of VirE2 and VirF to Cre recombinase. An equally elegant genetic assay for detection of nuclear-localizing proteins was developed in yeast. Here, the protein of interest is fused to a reporter construct mLexA-Gal4AD. Nuclear localization of the resulting chimeric protein results in both *lacZ* expression and activation of the yeast HIS3 gene and is thus scored by both the induction of β-galactosidase and the ability of the yeast transformants to form colonies on minimal media lacking histidine. Either of these assays may provide a more sensitive test for the subcellular location of MobA than the relatively blunt fluorescence experiments performed here.

The N-terminal 284 amino acids of MobA alone are sufficient for its DNA processing reactions and for RSF1010 conjugative transfer (Bhattacharjee and Meyer, 1993). Since MobA'-EGFP fusion proteins (N-terminal 216 amino acids) accumulated in the nucleus, it may be possible to test definitively the contribution of protein nuclear import to trans-kingdom conjugation frequencies by creating a further MobA' truncation variant that encodes the additional 68 amino acids required to restore MobA's DNA-processing functions (hopefully without loss of the nuclear localization ability). The yeast *S. cerevisiae* represents an ideal model eukaryotic organism for testing the biochemical aspects of trans-kingdom conjugation. Conjugation frequencies are easily determined by counting bacterial and yeast colonies on selective media and the genetic tractability of *S. cerevisiae* permits the application of the elegant genetic assays described above. Importantly, the MobA and TraI localization patterns were reproducible in yeast (M.W. Silby, C. Billington and J.A. Heinemann, personal communication). Thus, the results reported here are likely to apply broadly to eukaryotic organisms rather than specifically to cultured human cells.

Finally, it must not be taken for granted that the putative conjugative escort proteins accompany DNA all the way to the recipient cell nucleus or bacterial cytoplasm. It is probable that only a small number of protein-DNA conjugates enter the recipient cell during conjugation and these may unfortunately be below what is detectable in a genetic assay such as Cre/*lox* (Vergunst *et al.*, 2000). Donor cell proteins entering the recipient nucleus or cytoplasm likely remain there only transiently. Thus, the yeast HIS assay is not appropriate for detection of conjugatively transferred substrates (Rhee *et al.*, 2000). However, a sensitive genetic assay for this purpose should be conceivable, particularly, by the exploitation of genetic systems that, one triggered, maintain self-perpetuating expression. For example, putative conjugative escort proteins could be fused to the active portion of a hypothetical protein responsible for triggering excision and multiplication of a latent virus. For comparison, a similar assay was devised to

demonstrate conjugation-dependent transfer of RecA from donor to recipient bacteria (Heinemann, 1999).

To conclude, the results from these experiments give some insight into the possible role of the putative conjugative escort proteins TraI and MobA in trans-kingdom conjugation. While TraI's nuclear localizing ability fails to refute the hypothesis that nuclear transformation by putative escort proteins is a significant barrier to trans-kingdom exchange, the case of MobA could perhaps be used to argue the reverse. Certainly, more experimental work is required to test (i) the true (and possibly conditional) subcellular locale of MobA, (ii) the relevance of TraI's nuclear localization ability to IncPα trans-kingdom conjugation and (iii) the natural subcellular locations of other putative conjugative escort proteins such as TraI from the F plasmid, also demonstrated to partake in trans-kingdom conjugation (Sprague, 1991).

Should the nuclear localization of TraI indeed be found important for trans-kingdom conjugation, an interesting evolutionary question is raised; did TraI's nuclear localization potential arise by selection, this implying an importance of trans-kingdom conjugation for the evolution of a bacterial plasmid not known to have evolved any specific relationships with eukaryotic cells, or by chance? While it may seem unlikely that such a useful function pertinent specifically to eukaryotic proteins evolved within a bacterial protein, it should be noted that the requirements for nuclear localization within a protein are very minor. For example, the bacterial protein LexA encodes a five amino acid sequence both necessary and sufficient for its likely fortuitous nuclear localization when expressed in eukaryotic cells (Rhee *et al.*, 2000). Much is still to be done in elucidating the biochemical mechanisms of conjugative gene transfer and transmission from bacteria to eukaryotic cells.

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Chapter 6: Epilogue

The experiments presented in Chapters 3 and 4 demonstrate the ability of intracellular bacteria to meet and exchange plasmid DNA within animal cells, revealing the animal intracellular milieu as a permissive environment for gene exchange. This finding evokes a model for the simultaneous dissemination of virulence and antibiotic resistance within a niche protected from both antibiotics and the immune system (Chapter 3) and extends the variety of environments in which bacteria are known to exchange genes. Although the mechanism by which intracellular bacteria meet and exchange DNA remains indeterminate, the experiments presented in Chapter 4 provide a platform for further investigation.

The frequency of plasmid transmission between intracellular *S. typhimurium* was high compared with that in other environments, both natural (e.g. Christensen *et al.*, 1998; Dahlberg *et al.*, 1998; Anderson, 1975) and artificial (Chapter 3, table 2); however, the relevance of our experiments to natural biological environs remains to be explored. The reproduction of our intracellular plasmid transmission experiments within an animal model would indeed be convincing testimony for the relevance of our laboratory finding to bacterial evolution *in situ*. When compared with epithelial cells of the animal gut, cultured cells are poorly organized and undifferentiated (Nickerson *et al.*, 2001). Cultured cells lack microvilli and fail to secrete mucous or express a number of common epithelial receptors. At the other extreme, conditions within animal models are difficult to standardize, making animal modeling of gene transmission occurring specifically *within* cells potentially very problematic. However, a recently described novel method for cultivation of INT-407 cells as suspended 3D tissue assemblies may better resemble an animal model without the associated technical difficulties (Nickerson *et al.*, 2001). In contrast to 2D INT-407 cells cultured in plastic dishes, 3D cells were polarized, expressed epithelial markers, mucin and extracellular matrix proteins, and underwent differentiation to form tight junctions, desmosomes and microvilli. Subsequent comparisons of *S. typhimurium* invasion of 2D and 3D INT-407 cells

revealed that bacteria poorly bind to, and invade, cells more closely resembling those in the natural gut environment. In contrast, 2D cells internalized numerous bacteria with extensive concomitant damage to the cell surface and integrity. Furthermore, bacterial invasion of 2D cultured cells triggered a 60% increase in apoptosis whereas 3D cells showed little damage and no increase in apoptosis upon infection. Thus, it is possible that the intracellular conjugation experiments presented herein grossly over-estimate the frequency of bacterial meeting and gene exchange within natural epithelia. However, *S. typhimurium* and numerous other enteric pathogens preferentially traverse the epithelial barrier via entry into a particular cellular subtype called the M-cell (for a review see Finlay and Cossart, 1997). While physical association between gut bacteria within normal epithelial cells may occur rarely, it is possible that M-cells represent an environment more permissive for interspecies meeting and intracellular gene exchange. However, the lack of a continuous M-cell line (Nickerson *et al.*, 2001) prevents test of this possibility at present.

The plasmids used in our experiments, F and RP4, express conjugation genes constitutively and are thus transmitted between bacteria at high frequencies. While useful as models, F and RP4 are poorly representative of other natural conjugative plasmids, which are typically repressed for conjugative functions and are triggered to reproduce infectiously only under certain favourable conditions (Ghigo, 2001; Lundquist and Levin, 1986). However, since repression studies are typically done in artificial environments, the generality of repression is unknown. The potential for intracellular transmission of natural *S. typhimurium* HMEs, for example, the conjugative virulence plasmid pSLT (Ahmer *et al.*, 1999), is thus worthy of investigation. An interesting question is whether bacterial gene transmission may occur more frequently upon ‘sensing’ of environments where the acquisition of new traits (or from the point of view of the HME, epidemic infectious reproduction) may be of benefit. Conjugative transmission of pSLT and other F-like plasmids is subject to a number of potentially ‘environment-sensitive’ regulatory mechanisms (Camacho and Casadesús, 2002). Conjugal transfer is repressed by Dam

methylase, a sensor of DNA replication and DNA damage: methylation of adenine within specific GATC sites results in increased expression of *finP* mRNA. Antisense binding by *finP* mRNA prevents translation of mRNA encoding the conjugative activator protein, TraJ. Conversely, conjugative transfer is derepressed by the leucine-responsive regulatory protein (Lrp), which binds upstream of *traJ* and activates its transcription. Since Lrp acts upstream of Dam methylase, Lrp positive regulation of *traJ* overrides its negative regulation by methylation. Although the conditions triggering Lrp expression in this instance are unclear, it is likely that Lrp responds positively to high environmental nutrient concentrations (Camacho and Casadesús, 2002). Interestingly, Lrp and Dam methylase act in concert to control the epigenetic expression of a number of fimbrial biosynthesis genes in *E. coli* (van der Woude *et al.*, 1996; van der Woude and Low, 1994; Blyn *et al.*, 1989; van der Woude *et al.*, 1998; Braaten *et al.*, 1992). Pertinent to this discussion, Lrp and Dam methylase regulate the expression of the *S. typhimurium pef* fimbrial genes encoded on pSLT (Nicholson and Low, 2000). Thus, there is precedent for animal cell-associated, Lrp/Dam methylase-mediated, induction of genes encoded on the virulence plasmid. The complexity of these regulatory loops makes it difficult to predict *a priori* whether pSLT conjugative transfer is likely to be positively or negatively regulated, if at all, within the animal cell environment. This question awaits experimental test.

The vectors of bacterial gene exchange within animal cells may not be limited to conjugative plasmids. *S. typhimurium* (and many other enteric pathogens) harbor numerous lysogenic phage (Schicklmaier and Schmieger, 1995), many of them encoding virulence determinants (Miao and Miller, 1999; Mirold *et al.*, 1999; Cheetham and Katz, 1995; Hansen-Wester *et al.*, 2002b; a). Our preliminary data (not shown) indicated that lysogenic phage could be induced and recovered from bacteria residing within cultured cells. Thus, whether or not phage can mediate transfer of genes between intracellular bacteria by transduction is of interest. With reference to the previous discussion, lytic conversion of lysogenic phage typically occurs in response to RecA induction in times of host cellular stress (reviewed by

Walker, 1996). Thus, the intracellular environment may be particularly permissive for phage-mediated gene transfer and this would be an interesting topic for further investigation.

Another potential mechanism of intracellular gene transfer, both between bacteria and from bacteria to the host cells they occupy, is fusion of cellular membranes with the outer membrane buds, or 'blebs', released from gram-negative bacteria (Kaduragamuwa and Beveridge, 1995; 1996; Dorward and Garon, 1990; Dorward *et al.*, 1989; Li *et al.*, 1998). 'Blebs' typically contain linear and plasmid DNA, RNA and lytic enzymes and it is believed that the packaging of proteins and DNA into blebs occurs in a regulated fashion, possibly to prevent degradation or dilution of molecules intended for neighboring bacterial species. Blebbing mediates both the killing of competing bacteria (Li *et al.*, 1998) and the transfer of genes to others (Dorward and Garon, 1990; Dorward *et al.*, 1989). While blebbing appears to be a natural phenomenon in most if not all gram-negative bacteria, the frequency of blebbing is significantly increased by exposure of bacteria to membrane-perturbing antibiotics such as gentamicin (Kaduragamuwa and Beveridge, 1995). It is tempting to speculate that antibiotic treatment may potentiate bleb-mediated gene transfer in and around the animal cell environment (Heinemann, 1999). Curiously, intracellular *S. typhimurium* has been shown to release large amounts of LPS, in the form of SCV-derived membrane vesicles, into the host cell milieu (Garcia-del Portillo *et al.*, 1997). While the mechanism of LPS transfer into the SCV membrane has not been determined, it is possible that this also occurs in a blebbing-like fashion.

The above examples all represent unexplored mechanisms by which bacteria may exchange genes within animal cells. Of equal interest to us is the potential for gene transfer from bacteria to the animal cells within which they reside. As discussed in earlier chapters, gene transfer from bacteria to animal cells has been demonstrated to occur by various means: when lysed intracellularly, bacteria release plasmid DNA, which is subsequently transferred to the host cell nucleus

and expressed (Courvalin *et al.*, 1995; Grillot-Courvalin *et al.*, 1998). Further, extracellular bacteria have been shown to transfer DNA to animal cells by conjugation (Kunik *et al.*, 2001; Waters, 2001). The possibility that the T4S systems co-opted for protein transfer to animal cells during pathogenesis might also mediate gene transfer to the host cells within which they reside has yet to be exhaustively explored. The tendency of intracellular pathogens to rapidly kill host cells makes the detection of successful gene transfer problematic (data not shown). However, one intracellular pathogen whose potential DNA transfer ability is worthy of further study is *H. pylori*. *H. pylori* translocate at least one protein, CagA, into cultured epithelial cells by T4S (Christie, 1997). Interestingly, the *H. pylori* T4S system encodes a 'coupling protein' (Christie and Vogel, 2000); proteins from this class are essential for conjugative DNA transfer and potentially adapt the T4S apparatus for translocation of DNA substrates (Llosa *et al.*, 2002). While *H. pylori* is not unique in its retention of the coupling protein, and this in itself is not evidence that *H. pylori* does or even once did transfer DNA via its T4S system (since the coupling protein may also be essential, in some cases, for secretion of proteins across the inner membrane), the discovery of two putative relaxases encoded elsewhere in the *H. pylori* genome is indeed curious (Llosa *et al.*, 2002). That *H. pylori* was found to transfer plasmid DNA by an as yet genetically indeterminate, conjugation-like mechanism (Kuipers *et al.*, 1998), incites speculation that *H. pylori* may be capable of T4S-mediated plasmid transfer to other bacteria and maybe even human cells.

A functional relationship between pathogenesis and DNA transfer is an intriguing possibility. However, much of the biochemistry of bacterial conjugation still remains to be defined. How does DNA traverse the cellular membranes separating parental cytoplasms? What is the extent of concomitant protein transfer? How do transferred proteins contribute to DNA transmission and how do the biochemical requirements for DNA transmission to eukaryotic and prokaryotic recipients differ? An example is the differential requirement for DNA-binding proteins, such as VirE2, in conjugation: VirE2 is essential for T-DNA and RSF1010 transfer to plant cells

(e.g. Binns *et al.*, 1995) but dispensible for *vir*-mediated RSF1010 transfer to *Agrobacterium* (Beijersbergen *et al.*, 1992). On RP4, the functional equivalent of VirE2 is the primase TraC. TraC is also transferred to recipient bacteria during conjugation (Rees and Wilkins, 1990) but whether this protein is required for DNA transmission to eukaryotic cells, in a role similar to that of VirE2 (namely protection of transferred DNA against nucleolytic attack (Rossi *et al.*, 1996) and mediation of its efficient nuclear import (Ziemienowicz *et al.*, 2001)), is not known. That T-DNA transmission to eukaryotic cells requires bacterial proteins for efficient nuclear translocation of the T-complex is clear. Again, the applicability of this requirement to trans-kingdom conjugation in general is not yet known. The results presented here (Chapter 5) suggest that at least one putative 'pilot protein', TraI, possesses nuclear locating activity while another, MobA, appears to lack efficient means of nuclear translocation. The biological relevance of this finding awaits further test.

In summary, bacterial conjugation represents a robust and promiscuous mechanism of gene transmission, mediating gene transfer not only between bacterial species but from bacteria to a highly diverse range of eukaryotic cells. Bacterial conjugation occurring within animal cells may mediate the acquisition of antibiotic resistance genes by intracellular pathogens and virulence genes by normal flora in environments where such genes are concentrated and positively selected. Furthermore, the activity of the bacterial conjugation machinery within the intracellular environment may potentiate the transfer of bacterial DNA to animal cells. Indeed, such a finding may be harnessed for the benefit of genetic research: on-going research within our laboratory aims to exploit bacterial conjugative proteins to direct transferred DNA to the genetically-recalcitrant mitochondria.

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Appendix to Chapter 3

TABLE 1. Plasmid transmission occurs post-plating[a]

Expt	Replicate	Transmission frequency			
		Intracellular (RP4)	Post-plating (RP4)	Intracellular (F42::miniTn10Kn)	Post-plating (F42::miniTn10Kn)
1	1	2×10^{-4} (142)[b]	$\leq 1 \times 10^{-6}$ (0)	4×10^{-4} (60)	$\leq 7 \times 10^{-6}$ (0)
	2	3×10^{-4} (126)	$\leq 1 \times 10^{-6}$ (0)	7×10^{-4} (63)	$\leq 1 \times 10^{-5}$ (0)
	3	4×10^{-4} (128)	1×10^{-5} (4)	2×10^{-4} (45)	$\leq 7 \times 10^{-6}$ (0)
2	1	1×10^{-4} (43)	$\leq 2 \times 10^{-6}$ (0)	2×10^{-3} (123)	$\leq 7 \times 10^{-6}$ (0)
	2	1×10^{-4} (84)	$\leq 2 \times 10^{-6}$ (0)	9×10^{-4} (428)	$\leq 3 \times 10^{-6}$ (0)
	3	1×10^{-4} (48)	$\leq 2 \times 10^{-6}$ (0)	1×10^{-3} (335)	$\leq 4 \times 10^{-6}$ (0)
3	1	3×10^{-4} (382)	5×10^{-6} (10)	2×10^{-4} (272)	$\leq 2 \times 10^{-6}$ (0)
	2	4×10^{-4} (417)	1×10^{-5} (18)	6×10^{-4} (266)	$\leq 1 \times 10^{-6}$ (0)
	3	3×10^{-4} (523)	Replicate lost	4×10^{-4} (112)	$\leq 1 \times 10^{-6}$ (0)

4	1	1×10^{-4} (142)	3×10^{-6} (8)		
	2	8×10^{-5} (273)	1×10^{-5} (28)		
	3	1×10^{-4} (296)	2×10^{-6} (12)		
5	1	6×10^{-4} (194)	7×10^{-6} (7)		
	2	2×10^{-4} (492)	1×10^{-6} (1)		
	3	3×10^{-4} (515)	1×10^{-5} (12)		
6	1	4×10^{-4} (148)	$\leq 7 \times 10^{-7}$ (0)		
	2	2×10^{-4} (118)	$\leq 3 \times 10^{-6}$ (0)		
	3	5×10^{-5} (58)	8×10^{-6} (4)		
7	1	3×10^{-4} (1311)	4×10^{-7} (4)		
	2	6×10^{-4} (1539)	3×10^{-6} (8)		
	3	3×10^{-4} (1686)	7×10^{-6} (14)		
8	1	5×10^{-5} (121)	$\leq 3 \times 10^{-6}$ (0)		
	2	1×10^{-4} (147)	8×10^{-7} (4)		
	3	7×10^{-5} (150)	6×10^{-6} (4)		
Average		$2 \times 10^{-4} \pm 8 \times 10^{-5}$ (9083) [0 of 24][c]	$4 \times 10^{-6} \pm 2 \times 10^{-6}$ (138) [8 of 23]	$7 \times 10^{-4} \pm 3 \times 10^{-4}$ (1704) [0 of 9]	$3 \times 10^{-7} \pm 1 \times 10^{-7}$ (0) [9 of 9]

[a] In RP4 experiments, the donor and recipient were SL1344^{R} (RP4) and SL1344^{N}, respectively. In F experiments, the donor and recipient were 14028^{R}-P (F42::miniTn10Kn) and BA770, respectively. Plasmid transmission frequencies were calculated as the number of transconjugant colonies per limiting intracellular parent. Frequencies could not be calculated when recombinant colonies were not detected. The theoretical maximum frequencies were substituted for a conservative estimate of the average post-plating plasmid transmission frequency.

[b] The numbers in parentheses are the numbers of recombinant colonies observed, totaled in the final row.

[c] The numbers in square brackets are the proportion of replicates in which transconjugants were not detected.

TABLE 2. Binding and internalization of *invA*⁻ *S. typhimurium*

Strain	Binding[a]	Internalization[b]	% internalization[c]
SL1344	$4 \times 10^6 \pm 3 \times 10^5$	$2 \times 10^6 \pm 8 \times 10^5$	50
DinvA SL1344	$1 \times 10^6 \pm 2 \times 10^5$	$2 \times 10^4 \pm 4 \times 10^3$	2

[a]MDCK[1] monolayers grown in a 24 well culture tray were infected with *S. typhimurium* as described in 'Materials and Methods', Chapter 3. After 2 hours the cells were washed 3 times with PBS to remove non-adherent bacteria and the cells lysed with 0.5% deoxycholate in PBS. The lysates were diluted serially and titred on LB agar plates to determine the total number of bound and internalized bacteria. The titres represent the average cfu/well from 3 replicate wells.

[b]MDCK monolayers were infected with *S. typhimurium* as described above. After the washing steps and culture media was replaced with fresh culture media supplemented with gentamicin at 100μg/ml and incubated for an hour. The cells were then lysed and the intracellular bacteria titred. The titres represent the average cfu/well from 3 replicate wells.

[c]Internalized bacteria/bound (and internalized) bacteria.

[1] Experiments using Madin Darby Canine Kidney (MDCK) cells are reported since these experiments predated our acquisition of the INT-407 human intestinal cell line. Since both cell lines are regularly used in *S. typhimurium* invasion studies it is not expected that SL1344 binding and internalization differ significantly between the two cell lines.

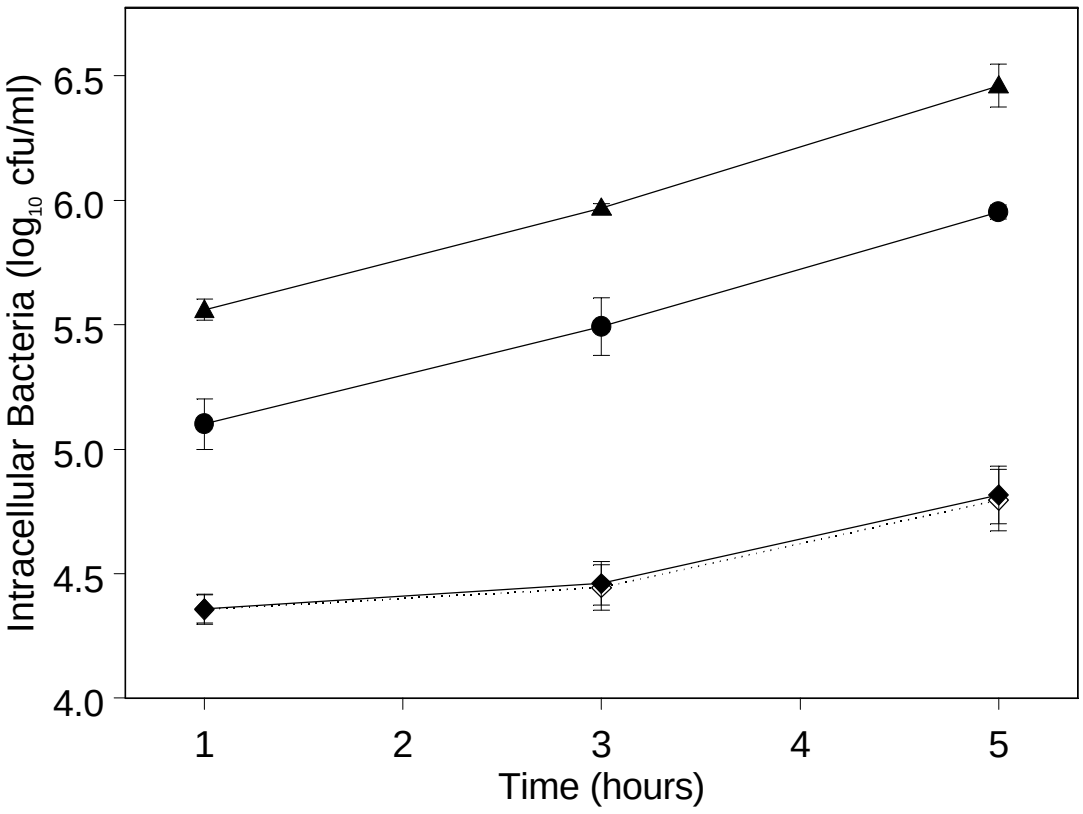

FIG. 1. Growth of parental and recombinant *S. typhimurium* within INT-407 cells over time[2]. We wished to exclude the possibility that the observed accumulation of intracellular recombinants over time was due to faster growth of the recombinant bacteria relative to the parental bacteria. Cultured cells were infected concurrently with donors, recipients and nascent recombinants. Nascent transconjugants were created immediately prior to invasion of tissue culture cells by mixing 10μl droplets of donor and recipient bacteria on LB agar plates for one hour. Exconjugants were harvested by scraping the bacteria from mating plates into 1ml of PBS. The bacteria were pelleted by centrifugation, resuspended in 1ml of PBS and 0.1ml of this was used to inoculate INT-407 cells grown in 24-well trays. The number of intracellular bacteria was determined over time as is described elsewhere (see Materials and Methods, Chapter 3). Donors, recipients and recombinants invaded

[2] Each value is an average based on one experiment performed in triplicate. Error bars indicate standard errors.

cells at an equivalent efficiency (data not shown). The x-axis (time) refers to the number of hours that the infected cells were incubated following the addition of gentamicin. Since the bacteria were allowed 2 hours to invade prior to addition of gentamicin, the bacteria recovered at the first time point were therefore intracellular for anywhere between 1 and 3 hours. Whereas recipients [$SL1344^{N}$ (?)] and donors [$SL1344^{R}$ (RP4) (?)] accumulated at an equal rate, recombinants accumulated at a slightly lesser rate [$SL1344^{N}$ (RP4) (υ)]. The number of intracellular recombinants at each time point was corrected for those that formed by recombination events that occurred after the bacteria had been introduced to the cells, ie. recombination events that occurred in the culture medium and between donors and recipients that had been taken into the same vacuole. The contribution of intracellularly-formed recombinants was determined by harvesting 10μl droplets of donor and recipient bacteria that had been plated separately on LB agar plates. These were mixed just prior to inoculation of the INT-407 cells. The corrected data (◇) superposes upon the uncorrected data (◆) therefore the accumulation of intracellularly-formed recombinants does not significantly effect measurement of the intracellular growth rate of recombinant bacteria. We conclude from this experiment that the accumulation of intracellular recombinants over time (Fig. 1, Chapter 3) was not due to faster intracellular growth of recombinants, formed early in the experiment, relative to the parental strains.

Appendix to Chapter 4.

A. Creation of a mutation in the *S. typhimurium ssrB* gene.

FIG.1. Primers for amplification of *ssrA* and *ssrB* sequences for creation of the *ssrB* mutagenesis plasmid pGCF13[a]

***ssrA*-forward**

5′ CC CTC TCT ATT TTC ATA CCG 3′

***ssrA*-reverse**

5′ T ACG CCT TTT CTC ACC CAG 3′

***ssrB*-*Bgl* II-forward**

5′ at tag atc tGC CTG TTG TGC ATA CGA GCC 3′
Bgl II

***ssrB*-reverse**

5′ TG ATA ACC TCC AGC GAA GCG 3′

[a]Annealing sequence is indicated in capitals. The positions of the primers within *ssrAB* are indicated in Figure 2. Template and primer DNA, dNTPs, Expand High Fidelity™ 10x buffer (with $MgCl_2$) and Expand High Fidelity™ Taq Polymerase were mixed in 50µl reaction volumes as per the recommendations of the manufacturer (Roche). PCR reactions were carried out in a PTC-150 Minicycler™ (MJ Research). The cycles for *ssrA* and *ssrB* amplification were as follows: 1 x [94°C, 2 minutes] 10x [94°C, 15 seconds; 53°C, 30 seconds; 72°C, 1 minute] 20x [94°C, 15 seconds; 53°C, 30 seconds; 72°C, 1 minute + a 20 second extension per cycle] 1x [72°C, 7 minutes].

FIG. 2. *ssrAB* sequence indicating the positions of the forward and reverse primers and relevant restriction sites[a]

```
     1          11         21         31         41         51
     |          |          |          |          |          |
   1 ACACAACTTT GTTTTTTTGT CTAAGTTTTC GCGGAGATTT TTTTTGACGT AATTAAATAT    60
  61 CAATAAGATA GAATGAGGGG AAGAAATCTA TTTCAGCGCC TATAGTGTGA TAACCTCCAG   120
                                                 ssrB-reverse
 121 CGAAGCGACC ACGTTGCGCC ACTGGGCAAG CTGTTTTTTC TGCCCGGCAG TGAGCTTTCC   180
 181 GGTGGTCACC AGGAACCATT TTCTCTGGGC GAACAGCTCG GGCGCCAGCA CAACTGGGGG   240
 241 CGAGGGAAGC ACATTGAGCC GCAGCTCTTG TCCGGTACGA GTCAACGTTT CCAGCCAGAT   300
 301 TTCACAGTGA TCCGAGAGAT TCCATCCGCT AATAAAGGCA TTGTTGCCAG GCGCTTTTCT   360
 361 GTCGCCTTCA AGGCAAAAAG AGGTGTAGGC AATAATGATG CCATCCAGTA TTTCGCGCAG   420
 421 CGTCATCACG GCGGGAAGAT GCGCGGACAC TTTGCTGCGC AACGGACGCA ATAAATGGGT   480
 481 CACCAGTTCT GCGCGGGGAT ATTCCCGGCC ACAGTCATAA ATCAACTGCC GCAGCGCGTC   540
 541 AATTCGCCCT TCGTGGAGAT GTTGAAGCAT CGTCTCCTGG ATCGTTATCC AGTTATCGCC   600
 601 CAGGCGAATA ACCGGACGCG ACAGTAAGGG TTTGACCTGG CTTATCGGTA CGCCTTTTCT   660
                                                 ssrA-reverse
 661 CACCCAGTCG AGAATGCTAA GCGCTTGTCG AATATCGTCA TCGCTGTATA AGCGATGTCC   720
 721 GCCATCAGTA CGCTGCGGTT TCAATAAACC ATAGCGTCGC TGCCATGCCC TTAGCGTGGC   780
 781 GGCATTGATA CCGCATAGTC TGGCAAATTC GCCGATAGAA TACGACATGG TAAAGCCCGT   840
 841 TAAATAAGGG GATTCTACTA TATCATGATC AAATGAAGAC TAAGGTAGTG ATAAATGGTG   900
 901 CGGCATACCA GGGCATCCGT ATGGATTTTG TCGATGATGA GCAATACTGC GTGGCGTAAG   960
 961 GCTCATCAAA ATATGACCAA TGCTTAATAC CATCGGACGC CCCTGGTTAA TACTCTATTA  1020
1021 ACCTCATTCT TCGGGCACAG TTAAGTAACT CTGTCACTTT ATGAACCTGT AGCTTTCTCA  1080
1081 TCATATTCAT CCGGTGTGTT TCGACGGTTT TTATACTGAT ATGTAGCTTT TCGCTGATCC  1140
1141 CATGATTGGT ATACCCCTCG TCAATAAGTT TAAGAACCTG ACGCTCGCGC AAAGTAAGCA  1200
1201 GTTGATGATT GGTCGTGTCA GCGTTTAATT CAGCCAGGAT AGCTTCCCGA TTCAACGTTG  1260
1261 GGTCAATGTA ACGCTTGTTT ACTGCTACTG TTTGCAATGC CGCTAACAGA ACTTGCTGAC  1320
1321 TACTGCTTTT TAAAACATAG CCATTAGCAC CTGCGGCTAA AGTTTTAATG GTCATATACT  1380
1381 CTTGTTGGTA TGCTGTGTAA ACCAGAATAT TCATTGCTGG CCAACGCTGA TGTAATTGAG  1440
1441 GAATGATATC CAGGCCATTG ATGCCAGGTA GACTAAGATC AAGGATAAGT ATGTCAGGCT  1500
1501 CGTATGCACA ACAGGCATTA TAAACCTCAA GACCATTTTT AACATGCTCT ACAATTTTAA  1560
      ssrB-BglII-forward
1561 AATGAGGCCA GGGTAATAAG GCATTCATAA TGCCGTTAAT GATGATTTCA TGATCGTCTA  1620
```

1621 CTAATAAGAT CTTATATTCT TTCATTTTGC TGCCCTCGCG AAAATTAAGA TAATA*TTAAG* 1680
***Bgl* II**
1681 *TAATGGTGTA GTTTTTGAAG ATCATACGTA TTTTCTGGCG TAAGTCGGTT AGTTCCTCCA* 1740
1741 *GCGCGATGAT TTTCCCCATT TTTACGCGAT TCTCAATGTC TATGACATAG CATACCAATT* 1800
1801 *CAGTCTGCCC TATTTGACCT AAACAGCCTT TTAATGTGTG AATTAACTGA TCGATTTTTT* 1860
1861 *CTCCAGCCGA TACGGCATTT TCAATATCAG CCAGCAAGAG GTCCAGTGAT TGGAAAATCT* 1920
1921 *TGCTATTAAT GACCATATCA TCTGTCGCCA GTAGCGCTGA GCAGCGACTC GGATCCTGCT* 1980
***Bam* HI**
1981 *CCTGTAGCTC TATATTTCGT AAAAGTTGGT ATTCTGCGGC AATACTGATG TAGCGAGCTA* 2040
2041 *AGGTAGCCAA TGTCACTGGT TTTGTAATGT AATGATGAAT CCCATTTTTT TTACAACGAT* 2100
2101 *GAATATCTTC TGTCGCTACG CTAGCGGATA GTGCCACAAA CATGCAGTCA GGATCTAAAT* 2160
2161 *TATTCGGCTC ATCATGCCAT AATCGTACAC ATTCAATACC ATCTATTTCT GGCATTCTAA* 2220
2221 *TGTCAATCAG TACTAAATCG AATCGCTGCT GTTGTGATAA AGTCAGAGCC TCGTTACTAC* 2280
2281 *TGGCGGCAAT AGTGACGTGT TGGCCCAGGC TGACAAGCAT TTTGCCGATG ATATCCCGAT* 2340
2341 *TAATATCGGC ATCATCAACC AAAAGAATCT GCAACTGCCA GGGTGGTAAC AAATTATTTA* 2400
2401 *TTACTGGCAT ATTTGGTGTA CACAATATTA ACTGTTGCGC CAGGTCGTAG AGTTTTCCGG* 2460
2461 *AGAAATACAA AAGCTCTGCG TTGAGAAGCG CATTTTGCTG GTGGGGTGGT TCACCGCGTA* 2520
2521 *TTCCCCAGCA AGCCAGTTGC CGATGCAGGC AGAACGGCGC TGACAGCGTC CCTTTAATTG* 2580
2581 *GTTGAGGCGG CTGGTATTCT TGTAAGGGTA ATACTAGCGA GACACAGGTT CCAACCCCGG* 2640
2641 *GGACACTTTT TAGTGTCAGA TTACCGCCCA TCATTTTAGC CAGGCTTGAC GCAATAGTCA* 2700
2701 *GTCCAATTCC TGTACCTTGC GAATTTGTGT CTGCTTGATA AAAAGCAGTA AAGATTTGAG* 2760
2761 *ACTGCTGCTG TATTTCAATC CCTTTACCGC TATCGCTAAC CAGAAATATT AATTGTTCCT* 2820
2821 *CATGACGCTT GACCGTCAGA CGTATCCCTC CGGTTTCGGT AAATTTTACC GCGTTCCCGA* 2880
2881 *GTAAATTAAC CAAAATTTGC CGTAAACGGA TACTGTCGGT ATGAAAATAG AGAGGGACAT* 2940
***ssrA*-forward**
2941 *GTTGACCGAC AAAAGTACGT AATGACAGTT TTTTGCTTTG CGCTGGCCCC TGGATGGTTT*..3000
3001 *GCATTGCCTG GTCCAGTAAC GGCAGTAACG CTGTTTCTTC CATATGTAAT GTGAAATGAC*..3060
3061 *CAGACTCGAT GCGTGAAAAA TCCAGCAGAT TATTAATAAT AGCTAACAAA GACAGTGTAC*..3120
3121 *AATTTCTGGC GGTATCAGCT AATCCTTGTT GCTCTATGTT TAAAGGGGTG GTTTGTAATA*..3180
3181 *ATTCAATTGC ACCGAGTACG CCATTCATCG GAGTACGTAA CTCATGACTT ATTACCGTAA*..3240
3241 *GATGAATGCT TTTACGTTTG TTAGCTCGCT CAGCGCGTTT TTTTGCTTCA TTTAGCGCCT*..3300
3301 *GGGTGCGCTC TGCGACTTTG TTTTCCAGAT TGTCGTATTG GACTTGTAGA GTATCAAGCA*..3360
3361 *GTTGGTTAAA AGCACCGGCA ATACTATCTA ATTCATCCAG TCGTTGTGCT GGTAAACGTG*..3420
3421 *TGCTCAGCGG TGCAGTTGCG GTTTTATTAA TGACATCGAC AAAACGCCAT AACGGTTTGG*..3480

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3481 CCAGTGAGCG ATGTAGTAAC CAGCAAAAAG CCGACGTCAT CAACACCAAT GCTGTTAATG..3540
3541 TAAAGGGGAT TTGTTGAAGG ATAATTTTTA AGATGCGATT ATGTAGATTA CCGTATGGGT..3600
3601 ACAGCGTAAC CAGACTCCAT CCGGGGCCAT GCAAGGTTGT GCGTAATATC AGAAATCCGG..3660
3661 GAATTTGCTG CCATCCATCA TGCAGCGTTA CATTTTCTAA CTGTGTACGT ATTTTTTGCG..3720
3721 GGATGTATGA AAACGGCAAT AAGTGGTTGT TTTGATCCAG CCATACTCGA ATACTATCAT..3780
3781 CTAATGGCAG GTGGCTCTTA GTAATGAGAT CGGGAAGTTT AACCGTCACC TCAAAAAATA..3840
3841 CGCCTTGCTG ATCGGCAACC GCAACGGAAG CGTGCCATCC TTTGCCGTTT ATGTATTCTG..3900
3901 GTTCACTCCA GTAAAACCCG GCATGGGTTG GGTATAAAGG AAAGCTTTTT CGCGTTAAAG..3960
3961 GCTGTAGAGT TGAATAATCT GAAGGGTTAT CAGTAGATAA TAACGAAATC TCATTTTTAT..4020
4021 GATTAAGAAT AAAACTATCG CGACGAAAGC TATTTTCATC GATATCAGAG GACTGCAGAA..4080
4081 AGAGACGGTG CTTCTCTCCG TTTAGCGTCG GCGTGCAATT TGAAGGACCG ACAGATAGAT..4140
4141 GCCGGCTCAC CTCAGGGAAA ATATCGTTAT GATGAATCTC AGTCGCTAAT GAGCATTGAT..4200
4201 ACATTAAATT TTTAGCGTCA CGTTCAGCTT CTTCAAACCG TTGATTGCTT AGTACAATAT..4260
4261 TCATCTCGGA TAGAACGGAT AAATCCTCTA TTATATGCTG CCGTTTCTGA ACCATTGATA..4340
4341 TATAAGCTGC GGTAAGCACA GATAGCAGCC AAATAATTAT TGTTGTTAAT AAAAATAAAA..4400
4401 AAGTTAGCCT GATTACTAAA GATGTTTGCA GCGTATTCTT GAGATTGAGC AAATTCATAA..4460
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[a]The S. *typhimurium* genome sequence was obtained from the Washington University School of Medicine Genome Sequencing Centre. St. Louis, WA USA (http://genome.wustl.edu/gsc/search/ftp.shtml). *ssrAB* sequence was obtained from Genbank (accession number Z95891). *ssrA* (4458-1676) and *ssrB* (1588-1007) complementary coding sequence is indicated in italics.

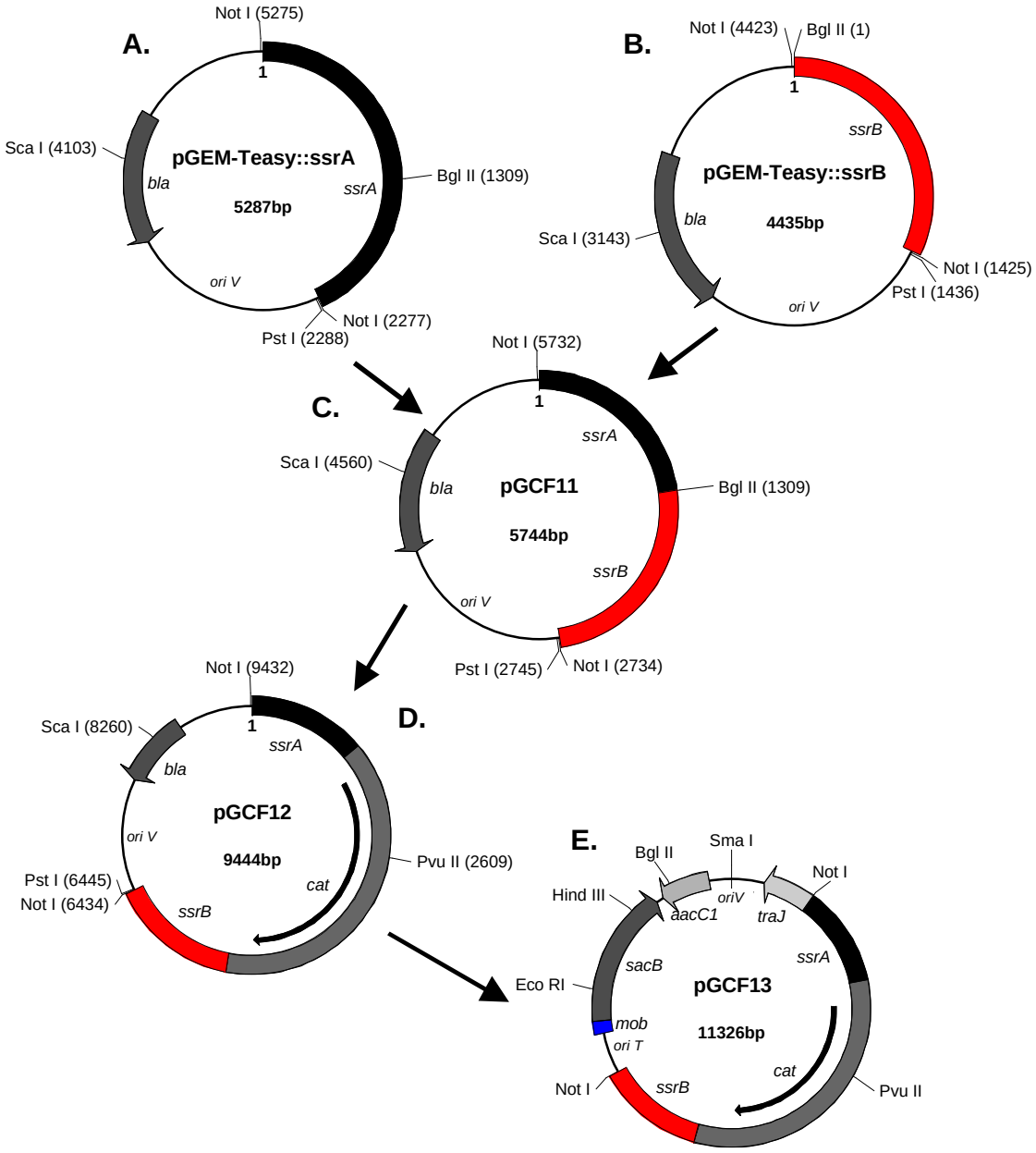

FIG.3. Construction of the *ssrB* mutagenesis plasmid pGCF13. A 2.269Kb fragment encoding *ssrAB* and flanking sequences was generated from *S. typhimurium* MS1868 chromosomal DNA by PCR using primers *ssrA*-forward and *ssrA*-reverse. The resulting product was ligated with the PCR cloning vector pGEM-Teasy (Promega) to create pGEM-Teasy::*ssrA* (A). A second fragment encoding a 1.417Kb 5'-truncated *ssrB* gene and downstream sequence was generated similarly using primers *ssrB-Bgl* II-forward and

ssrB-reverse, which created a *Bgl* II restriction site at the 5' end of the fragment. This product too was ligated with pGEM-Teasy to create pGEM-Teasy::*ssrB* (B). The orientations of the cloned fragments within pGEM-Teasy were confirmed by restriction analysis (data not shown). pGEM-Teasy::*ssrB* was digested with *Pst* I and *Bgl* II and the 1.5Kb fragment encoding 5' truncated *ssrB* was ligated with the 4.239Kb fragment of *Pst* I/*Bgl* II-digested pGEM-Teasy::*ssrA* to create pGCF11 (C). pGCF11 was linearized with *Bgl* II and ligated with a ~3.7Kb *Bam* HI fragment of the plasmid pHP45Ω-Cm encoding a chloramphenicol resistance (Cm^R) cassette. (Fellay *et al.*, 1987) The orientation of the Cm^R cassette within the resulting plasmid, pGCF12 (D), was determined by restriction analysis (data not shown). A 6.4Kb *Not* I fragment of pGCF12, encoding *ssrAB*::Cm, was ligated with the plasmid $pJQ200_{SK}$ (Quandt and Hynes, 1993)to create pGCF13 (E). The orientation of *ssrAB*::Cm within pGCF13 was not determined. pGCF13 was mobilized to *S. typhimurium* strain MS1868 from *E. coli* S17.1-λ pir by conjugation. Since the degradation products of sucrose (by the biochemical activity of sucrase, encoded by the *sucB* gene on pGCF13) are toxic to *S. typhimurium*, double cross-over recombination events (resulting in loss of the replication-deficient pGCF13 plasmid backbone) were selected by plating transconjugants on LB agar plates supplemented with chloramphenicol (20μg/ml) and 5% sucrose. Loss of the pGCF13 vector was confirmed by scoring putative recombinant bacteria for gentamicin sensitivity.

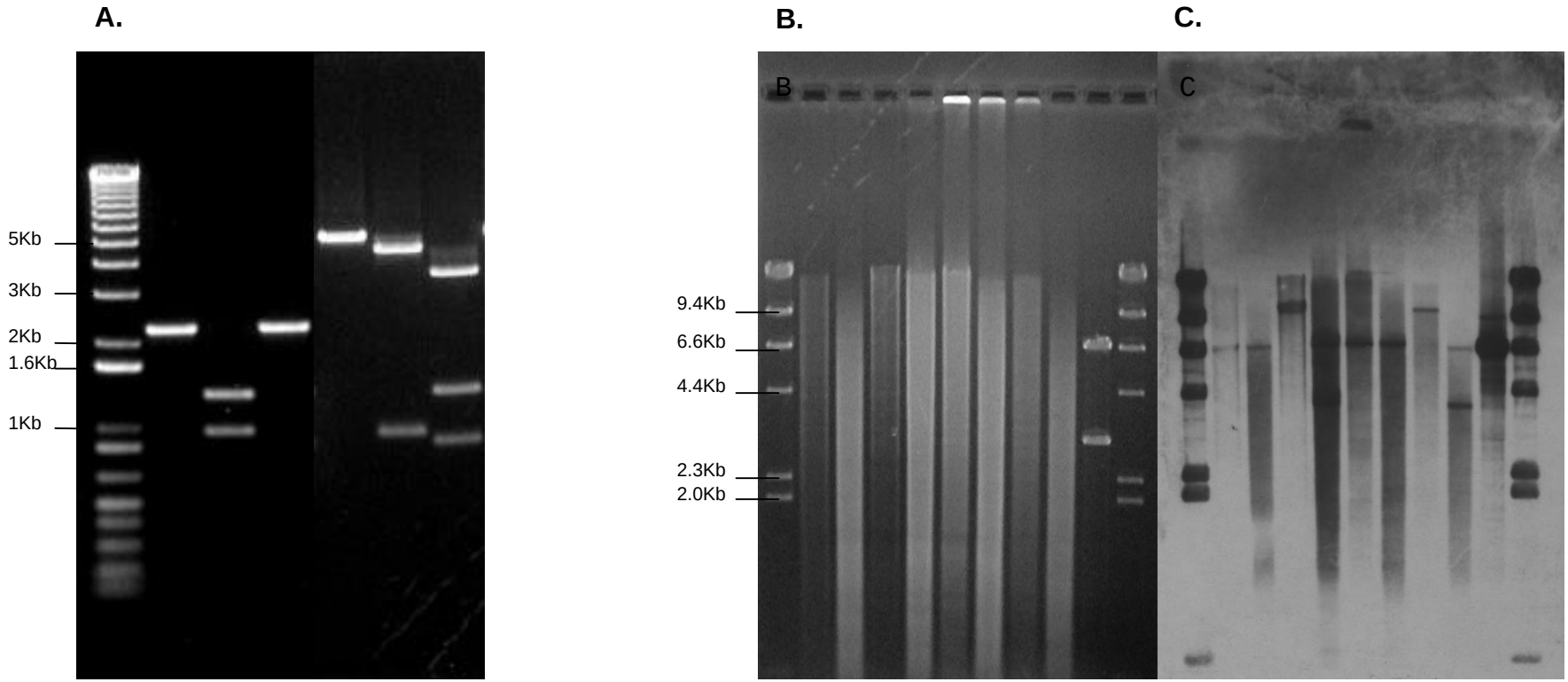

FIG.4. Confirmation of a chloramphenicol resistance cassette (*cat*) insertion within the *S. typhimurium* chromosomal gene *ssrB*.

Insertion of *cat* within *ssrB* in MS1868 was confirmed by PCR amplification of the *ssrAB* region with primers *ssrA*-forward and *ssrA*-reverse (A) and by Southern hybridization following transfer of the *ssrB*::Cm mutation to SR11^{R} by P22 transduction (C and D).

A. Lane 1, 1 Kb^{+} ladder (Invitrogen); Lane 2, MS1868 *ssrAB* PCR fragment, undigested; Lane 3, MS1868 *ssrAB* PCR fragment, *Bam* HI digest; Lane 4, MS1868 *ssrAB* PCR fragment, *Hin*d III digest; Lane 5, MS1868$_{ssrB::Cm}$ *ssrAB* PCR fragment, undigested; Lane 6, MS1868$_{ssrB::Cm}$ *ssrAB* PCR fragment, *Bam* HI digest; Lane 7, MS1868$_{ssrB::Cm}$ *ssrAB* PCR fragment *Hin*d III digest.

Amplification of MS1868 *ssrAB* DNA with primers *ssrA*-forward and *ssrB*-reverse produced the expected 2.249Kb product. Restriction digestion of this product with *Bam* HI (which cuts within *ssrA*) produced the expected 1.322Kb and 965bp fragments. Digestion with *Hin*d III, which does not cut within *ssrAB*, produced a fragment whose gel migration was indistinguishable from the undigested product. In contrast, the putative *ssrB*::Cm mutant produced a PCR product of approximately 6kb. Digestion of this with *Bam* HI produced the predicted 5Kb and 965bp products while digestion with *Hin*d III (whose restriction sites flank the *cat* cassette) produced the predicted 3.7Kb (*cat* cassette), 1.309Kb and 867bp flanking fragments.

B. Genomic digests for Southern hybridization. Lanes 1 and 11, λ *Hin*d III standard; Lane 2, MS1868^{R} *Pst* I digest; Lane 3, MS1868^{R} *Pst* I/*Pvu* II digest; Lane 4, MS1868$^{R}_{ssrB::Cm}$ *Pst* I digest; Lane 5, MS1868$^{R}_{ssrB::Cm}$ *Pst* I/*Pvu* II digest; Lane 6, SR11^{R} *Pst* I digest; Lane 7, SR11^{R} *Pst* I/*Pvu* II digest; Lane 8, SR11$^{R}_{ssrB::Cm}$ *Pst* I digest; Lane 9, SR11$^{R}_{ssrB::Cm}$ *Pst* I/*Pvu* II digest; Lane 10, pGCF12 *Not* I digest.

C. MS1868^{R} and SR11^{R} wild-type and *ssrB*::Cm genomic digests (C) probed by Southern hybridisation with the 6.5Kb *Not* I pGCF12 fragment encoding *ssrAB*::Cm. Digested genomic DNA was transferred to Hybond-N^{+} membrane (Amersham Pharmacia Biotech.) using a VacuGene^{TM} XL vacuum blotting system (Pharmacia LKR Biotechnology) according to the instructions of the manufacturer. Hybridization, probe labeling and detection were carried out using an ECL^{TM} Direct NA Labeling and Detection system (Amersham Life Science) according to the instructions of the manufacturer. Hybridization was performed overnight in a rotisserie oven and membranes exposed to Hyperfilm MP (Amersham Pharmacia Biotech.) for 10 minutes before development.

Digestion of *S. typhimurium* genomic DNA with *Pst* I produced the expected 6.7Kb wild-type hybridizing fragment and the predicted 10.4Kb hybridizing fragment from the *ssrB*::Cm mutants. Digestion with *Pst* I and *Pvu* II, which cuts exclusively within the *cat* insert, produced the expected unaltered 6.7Kb wild-type hybridizing fragment and the predicted 6.7Kb and 3.7Kb hybridizing fragments from the *ssrB*::Cm mutants.

B. Creation of an *S. typhimurium sipC* deletion mutant.

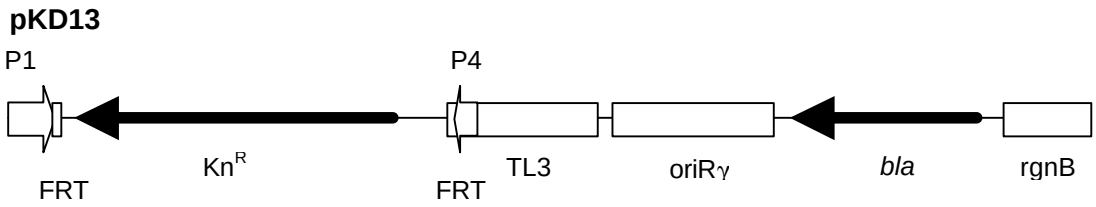

Fig. 5. Construction of *S. typhimurium sipC* deletion mutants [adapted from Datsenko *et al*. (Datsenko and Wanner, 2000)]. A kanamycin resistance marker (Kn^R) flanked by bacteriophage λ recombination sites (FRT) was amplified by PCR initiated at priming sites P1 and P4. Primers P1 and P4 encoded 5' 50nt overhanging extensions of sequence homologous to the ends of *sipC*. PCR products were purified by gel electrophoresis, digested with *Dpn* I, which cleaves contaminating (methylated) template DNA, and introduced into *S. typhimurium* strain MS1868 (pKD46) by transformation. pKD46 encodes the λ Red recombinase system (consisting of three genes: *g*, *b* and *exo*) which inhibits the host RecBCD exonuclease V whilst promoting homologous recombination at linear DNA ends. Expression of *g*, *b* and *exo* was induced by growth of the overnight culture in SOB supplemented with 10mM arabinose and 100μg/ml ampicillin prior to transformation. Homologous recombination between *sipC* and the 1.4Kb PCR fragment resulted in replacement of *sipC* with the Kn^R marker. The resulting MS1868 *sipC*::Kn mutant was cured of pKD46 by passage at 43°C. The sipC::Kn mutation was subsequently transferred to wild type *S. typhimurium* backgrounds by P22 transduction. Excision of the Kn^R marker was mediated by transformation of the resulting transductants with the pCP20 helper plasmid encoding the λ FLP recombinase, which acts upon the directly repeated FRT sites. *sipC* deletion mutants were cured of pCP20 by passage at 43°C.

FIG. 6. Primers for amplification of *sipBCD* sequences and creation of an *S. typhimurium sipC* deletion mutant[a]

***sipB*-forward**

5′ ATA ATC TGT CAA ATG TCG CCC 3′

***sipD*-reverse**

5′ CT GGT AAT AAC CAG CCT CCC 3′

SipC-P1

5′ *ttc gcg ttt tat tct gcg cca gag tcg cgc ata aaa act gcc aaa ata aa* GTG TAG GCT GGA GCT GCT TC 3′

SipC-P4

5′ *tag cag cga gtg cgg atg ctt tcg act ggt taa tgc tct* ATT CCG GGG ATC CGT CGA CC 3′

[a]Annealing sequence is indicated in capitals. Sequences within primers SipC-P1 and SipC-P4 complementary to the template plasmid pKD13 are indicated in capitals. Sequences within primers SipC-P1 and SipC-P4 derived from *sipC* flanking sequence are indicated in italics. The positions of the primers within *sipBCD* are indicated in Figure 6. Template and primer DNA, dNTPs, Expand High Fidelity™ 10x buffer (with $MgCl_2$) and Expand High Fidelity™ Taq Polymerase were mixed in 50µl reaction volumes as per the recommendations of the manufacturer (Roche). PCR reactions were carried out in a PTC-150 Minicycler™ (MJ Research). The cycles for amplification of KD13 with *sipC* homology extensions were as follows: 1 x [94°C, 2 minutes] 10x [94°C, 15 seconds; 57°C, 30 seconds; 72°C, 1 minute] 20x [94°C, 15 seconds; 57°C, 30 seconds; 72°C, 1 minute + a 20 second extension per cycle] 1x [72°C, 7 minutes]. Amplification of *S. typhimurium* genomic DNA with primers *sipB*-forward and *sipD*-reverse was carried out as described above except with an annealing temperature of 55°C and an extension time of two minutes.

Fig. 7. *sipBCD* sequence indicating the positions of the forward and reverse primers and primer extensions[a]

```
     1          11         21         31         41         51
     |          |          |          |          |          |
   1 ATGGTAAATG ACGCAAGTAG CATTAGCCGT AGCGGATATA CCCAAAATCC GCGCCTCGCT    60
  61 GAGGCGGCTT TTGAAGGCGT TCGTAAGAAC ACGGACTTTT TAAAAGCGGC GGATAAAGCT   120
 121 TTTAAAGATG TGGTGGCAAC GAAAGCGGGC GACCTTAAAG CCGGAACAAA GTCCGGCGAG   180
 181 AGCGCTATTA ATACGGTGGG TCTAAAGCCG CCTACGGACG CCGCCCGGGA AAAACTCTCC   240
 241 AGCGAAGGGC AATTGACATT ACTGCTTGGC AAGTTAATGA CCCTACTGGG CGATGTTTCG   300
 301 CTGTCTCAAC TGGAGTCTCG TCTGGCGGTA TGGCAGGCGA TGATTGAGTC ACAAAAAGAG   360
 361 ATGGGGATTC AGGTATCGAA AGAATTCCAG ACGGCTCTGG GAGAGGCTCA GGAGGCGACG   420
 421 GATCTCTATG AAGCCAGTAT CAAAAAGACG GATACCGCCA AGAGTGTTTA TGACGCTGCG   480
 481 ACCAAAAAAC TGACGCAGGC GCAAAATAAA TTGCAATCGC TGGACCCGGC TGACCCCGGC   540
 541 TATGCACAAG CTGAAGCCGC GGTAGAACAG GCCGGAAAAG AAGCGACAGA GGCGAAAGAG   600
 601 GCCTTAGATA AGGCCACGGA TGCGACGGTT AAAGCAGGCA CAGACGCCAA AGCGAAAGCC   660
 661 GAGAAAGCGG ATAACATTCT GACCAAATTC CAGGGAACGG CTAATGCCGC CTCTCAGAAT   720
 721 CAGGTTTCCC AGGGTGAGCA GGATAATCTG TCAAATGTCG CCCGCCTCAC TATGCTCATG   780
                           ---------->
                           sipB-forward
 781 GCCATGTTTA TTGAGATTGT GGGCAAAAAT ACGGAAGAAA GCCTGCAAAA CGATCTTGCG   840
 841 CTTTTCAACG CCTTGCAGGA AGGGCGTCAG GCGGAGATGG AAAAGAAATC GGCTGAATTC   900
 901 CAGGAAGAGA CGCGCAAAGC CGAGGAAACG AACCGCATTA TGGGATGTAT CGGGAAAGTC   960
 961 CTCGGCGCGC TGCTAACCAT TGTCAGCGTT GTGGCCGCTG TTTTTACCGG TGGGGCGAGT  1020
1021 CTGGCGCTGG CTGCGGTGGG ACTTGCGGTA ATGGTGGCCG ATGAAATTGT GAAGGCGGCG  1080
1081 ACGGGAGTGT CGTTTATTCA GCAGGCGCTA AACCCGATTA TGGAGCATGT GCTGAAGCCG  1140
1141 TTAATGGAGC TGATTGGCAA GGCGATTACC AAAGCGCTGG AAGGATTAGG CGTCGATAAG  1200
1201 AAAACGGCAG AGATGGCCGG CAGCATTGTT GGTGCGATTG TCGCCGCTAT TGCCATGGTG  1260
1261 GCGGTCATTG TGGTGGTCGC AGTTGTCGGG AAAGGCGCGG CGGCGAAACT GGGTAACGCG  1320
1321 CTGAGCAAAA TGATGGGCGA AACGATTAAG AAGTTGGTGC CTAACGTGCT GAAACAGTTG  1380
1381 GCGCAAAACG GCAGCAAACT CTTTACCCAG GGGATGCAAC GTATTACTAG CGGTCTGGGT  1440
1441 AATGTGGGTA GCAAGATGGG CCTGCAAACG AATGCCTTAA GTAAAGAGCT GGTAGGTAAT  1500
1501 ACCCTAAATA AAGTGGCGTT GGGCATGGAA GTCACGAATA CCGCAGCCCA GTCAGCCGGT  1560
1561 GGTGTTGCCG AGGGCGTATT TATTAAAAAT GCCAGCGAGG CGCTTGCTGA TTTTATGCTC  1620
1621 GCCCGTTTTG CCATGGATCA GATTCAGCAG TGGCTTAAAC AATCCGTAGA AATATTTGGT  1680
1681 GAAAACCAGA AGGTAACGGC GGAACTGCAA AAAGCCATGT CTTCTGCGGT ACAGCAAAAT  1740
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1741 GCGGATGCTT CGCGTTTTAT TCTGCGCCAG AGTCGCGCAT AAAAACTGCC AAAATAAAGG 1800

***sipBC* extension on primer SipC-P1**

1801 GAGAAAAATA TGTTAATTAG TAATGTGGGA ATAAATCCCG CCGCTTATTT AAATAATCAT 1860
1861 TCTGTTGAGA ATAGTTCACA GACAGCTTCG CAATCCGTTA GCGCTAAAGA TATTCTGAAT 1920
1921 AGTATTGGTA TTAGCAGCAG TAAAGTCAGT GACCTGGGGT TGAGTCCTAC ACTGAGCGCG 1980
1981 CCTGCGCCAG GGGTATTAAC GCAAACCCCC GGAACGATCA CGTCCTCTTT AAAAGCCAGT 2040
2041 ATTCAAAATA CCGACATGAA TCAGGATTTG AATGCTCTGG CAAATAATGT CACGACTAAA 2100
2101 GCGAATGAGG TTGTGCAAAC CCAGTTACGC GAGCAGCAGG CAGAAGTCGG AAAGTTTTTT 2160
2161 GATATTAGCG GAATGTCTTC CAGTGCCGTT GCGCTGTTGG CTGCCGCGAA TACGTTAATG 2220
2221 CTGACGTTGA ACCAGGCTGA TAGCAAACTG TCTGGTAAGT TGTCATTAGT CAGTTTTGAT 2280
2281 GCAGCTAAAA CGACGGCAAG CTCCATGATG CGCGAAGGGA TGAATGCGTT GTCCGGTAGT 2340
2341 ATTTCCCAGA GCGCGCTTCA GTTGGGGATC ACTGGCGTGG GCGCCAAACT GGAATATAAG 2400
2401 GGGCTGCAGA ATGAAAGAGG CGCGCTTAAA CATAATGCCG CGAAGATCGA TAAACTGACC 2460
2461 ACTGAAAGCC ACAGTATTAA AAACGTGCTG AACGGGCAGA ATAGCGTCAA ACTCGGTGCT 2520
2521 GAAGGCGTCG ATTCTCTGAA ATCGTTAAAT ATAAGAAAAC CGGTACCGAT GCGACGAAAA 2580
2581 ATCTTAATGA TGCGACGCTT AAATCTAATG CCGGAACCAG CGCCACGGAA AGTCTGGGTA 2640
2641 TTAAAGACAG TAATAAACAA AGTCTCCCTG AACATCTATA TCTTGTCGAA ACGTCTTGAG 2700
2701 TCTGTCGAAT CCGATATTCG TCTTGAGCAG AATTACATGG ATATTACCCG AATCGATAGC 2760
2761 GCGCAAGATG CAGATGACGG GCGATCTGAT TATGAAGAAC TCGGTCACGG TCGGTGGTAT 2820
2821 TGCAGGGGCG TCCGGGCAGT ACGCCGCTAC TCAGGTAACG TTTCCGAGCA GCAAATTAGC 2880
2881 CAGGTGAATA ACCGGGTTGC CAGCACCGCA TCGGACGAAG CCCGTGAAAG TTCACGTAAA 2940
2941 TCGACCAGCC TGATTCAGGA AATGCTGAAA ACAATGGAGA GCATTAACCA GTCGAAAGCA 3000

***sipC* extension on primer SipC-P4**

3001 TCCGCACTCG CTGCTATCGC AGGCAATATT CGCGCTTAAT CTGAAAGGTC ATCTATACGC 3060
3061 CATCATGGGT GTGATTTAAT CGCGCTCCTG ATGGCGAACT GGGGATATTA TGCTTAATAT 3120
3121 TCAAAATTAT TCCGCTTCTC CTCATCCGGG GATCGTTGCC GAACGGCCGC AGACTCCCTC 3180
3181 GGCGAGCGAG CACGTCGAGA CTGCCGTGGT ACCGTCTACC ACAGAACATC GCGGTACAGA 3240
3241 TATCATTTCA TTATCGCAGG CGGCTACTAA AATCCACCAG GCACAGCAGA CGCTGCAGTC 3300
3301 AACGCCACCG ATCTCTGAAG AGAATAATGA CGAGCGCACG CTGGCGCGCC AGCAGTTGAC 3360
3361 CAGCAGCCTG AATGCGCTGG CGAAGTCCGG CGTGTCATTA TCCGCAGAAC AAAATGAGAA 3420
3421 CCTGCGGAGC GCGTTTTCTG CGCCGACGTC GGCCTTATTT AGCGCTTCGC CTATGGCGCA 3480
3481 GCCGAGAACA ACCATTTCTG ATGCTGAGAT TTGGGATATG GTTTCCCAAA ATATATCGGC 3540
3541 GATAGGTGAC AGCTATCTGG GCGTTTATGA AAACGTTGTC GCAGTCTATA CCGATTTTTA 3600
3601 TCAGGCCTTC AGTGATATTC TTTCCAAAAT GGGAGGCTGG TTATTACCAG GTAAGGACGG 3660

***sipD*-reverse**

```
3661 TAATACCGTT AAGCTAGATG TTACCTCACT CAAAAATGAT TTAAACAGTT TAGTCAATAA  3720
3721 ATATAATCAA ATAAACAGTA ATACCGTTTT ATTTCCAGCG CAGTCAGGCA GCGGCGTTAA  3780
3781 AGTAGCCACT GAAGCGGAAG CGAGACAGTG GCTCAGTGAA TTGAATTTAC CGAATAGCTG  3840
3841 CCTGAAATCT TATGGATCCG GTTATGTCGT CACCGTTGAT CTGACGCCAT TACAAAAAAT  3900
3901 GGTTCAGGAT ATTGATGGTT TAGGCGCGCC GGGAAAAGAC TCAAAACTCG AAATGGATAA  3960
3961 CGCCAAATAT CAAGCCTGGC AGGCGGGTTT TAAAGCGCAG GAAGAAAATA TGAAAACCAC  4020
4021 ATTACAGACG CTGACGCAAA AATATAGCAA TGCCAATTCA TTGTACGACA ACCTGGTAAA  4080
4081 AGTGCTGAGC AGTACGATAA GTAGCAGCTG GAAACCGCCA AAAGCTTCCT GCAAGGATAA  4140
4141 CAGAAGAGGA TATTAA
```

[a]*S. typhimurium sipBCD* sequence was obtained from Genbank (accession numbers AE008831 and AE006468). *sipB* (1-1782), *sipC* (1810-3039) and *sipD* (3091-4156) coding sequence is indicated in italics.

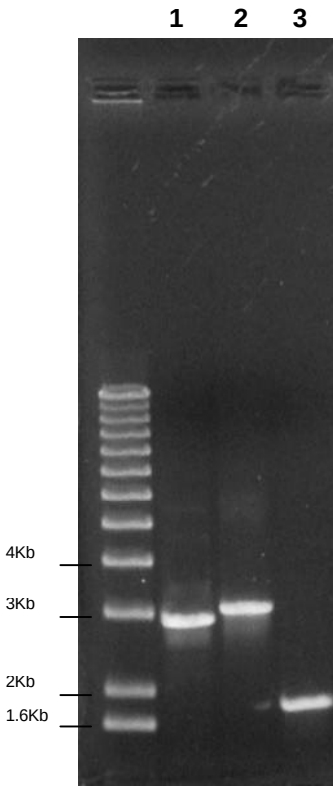

FIG. 8. Confirmation of *sipC* deletion. Deletion of *sipC* in *S. typhimurium* mutants was confirmed by PCR. Amplification of $SR11^{N}$ genomic DNA with primers *sipB*-forward and *sipD*-reverse produced the expected 2.907Kb wild-type *sipBCD* product (lane 1). In contrast, amplification of genomic DNA from the mutants $SR11^{N}_{sipC::km}$ and $SR11^{N}_{\Delta sipC}$ produced the predicted 3Kb and 1.684Kb products, respectively (lanes 2 and 3).

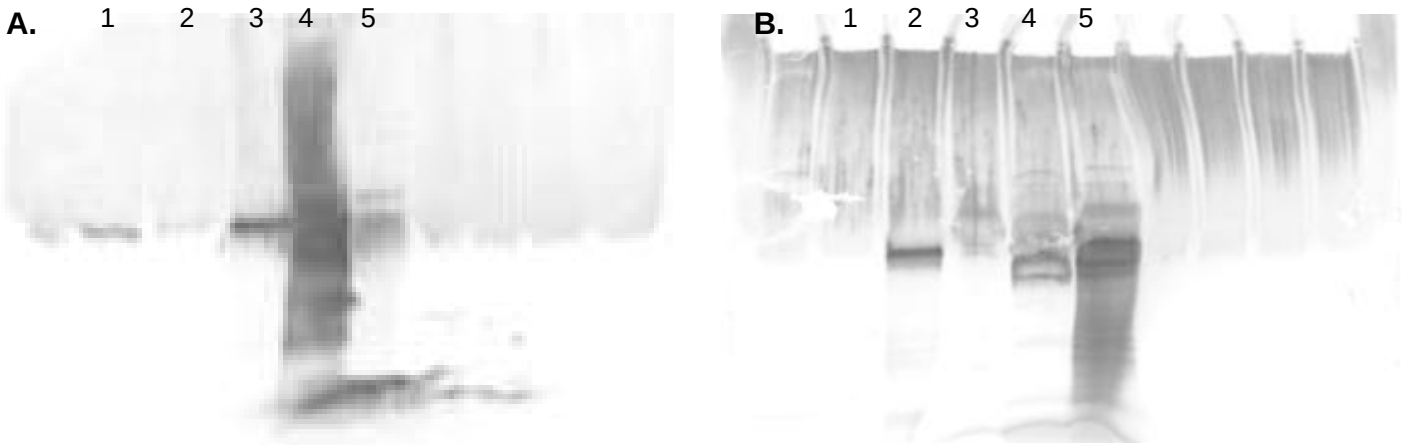

FIG.9. SipC and IpaC proteins are secreted by S. typhimurium. 50ml supernatants from *S. typhimurium* overnight cultures were filtered through a 0.2μm filter (millipore) and the proteins precipitated with TCA. (Osiecki *et al.*, 2001) Protein pellets were washed with acetone and resuspended in 100μl of nanopure water. The proteins in 40μl aliquots were separated on 8-16% SDS-PAGE gels (Gradipore) and then transferred onto Sequi-Blot™ PVDF membranes (Bio-Rad) according to the instructions of the manufacturer. The presence of SipC and IpaC was detected with polyclonal antiserum raised against SipC (A) and IpaC (B), respectively. Membranes were probed for primary antibody binding by incubation with a secondary goat anti-rabbit IgG (H+L) antibody conjugated to alkaline phosphatase (AP, Bio-Rad). Alkaline phosphatase activity was detected with an AP Conjugate Substrate Kit (Bio-Rad) according to the instructions of the manufacturer. Lane 1, purified SipC protein; lane 2, purified IpaC protein; lane 3, $SR11^{N}$; lane 4, $SR11^{N}_{\Delta sipC}$ ($sipC^{+}$); lane 5, $SR11^{N}_{\Delta sipC}$ ($ipaC^{+}$). The purified SipC (43kDa) and IpaC (40kDa) proteins and antibodies were a gift from Wendy Picking (University of Kansas). A strong αIpaC signal was observed in the $SR11^{N}_{\Delta sipC}$ ($ipaC^{+}$) sample (B, lane 5), although extensive protein shearing was evident. Similarly, the $SR11^{N}_{\Delta sipC}$ ($sipC^{+}$) sample gave a strong αSipC signal (A, lane 4). A small amount of cross-reactivity of the αIpaC and αSipC antibodies to SipC and IpaC, respectively, was observed (A, lane 5 and B, lane 4). In contrast, no αIpaC signal was observed in the wild-type $SR11^{N}$ sample (B, lane 3) whereas αSipC gave a strong signal (A, lane 3).

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Appendix to Chapter 5.

FIG. 1. Primers for amplification of *traI* and *mobA* sequences[a]

MobF1
5′ *ccc aag ctt ggg* **ATG** GCG ATT TAT CAC CTT ACG G 3′
*Hin*d III

MobF1-human
5′ *cc caa gct tgg* **ATG** GCG ATT TAT CAC CTT ACG G 3′
*Hin*d III

MobR1-human
5′ *cg cgg atc ctg gcg* [b]CAT GCT GAA ATC TGG 3′
Bam HI

MobR2-human
5′ *cg cgg atc ctc gcg* TTC CAT CTC CAC CA 3′
Bam HI

TraF1-B
5′ *aga aag ctt* [c]**ATG** ATT GCC AAG CAC GTC 3′
*Hin*d III

TraF1-B-human
5′ *ag aaa gct tat* [c]**ATG** ATT GCC AAG CAC GTC 3′
*Hin*d III

TraR1-human
5′ *ag agg atc ctt* TCT ACT CCT ACC TCG 3′
Bam HI

TraR2-human
5′ *ag agg atc ctt* CTC CTT GAA CTC TGC 3′
Bam HI

TraF2-human
5′ *tac att aag ctt* [d]**atg** GCT ATC AAA CTG CTG GGC G 3′
*Hin*d III

MobF2-human
5′ *tga gtc aag ctt* [d]**atg** GGC CGG GGC ATC CGC AC 3′
*Hin*d III

Modified MobR1-human
5′ *ct gtc tgg atc ctg* [e]***tcg*** CAT GCT GAA ATC TGG 3′
*Hin*d III

[a]Italicized bases are not derived from the *mobA* or *traI* coding sequence. These were introduced either to create a restriction site, aid restriction digestion of PCR products or to

correct the reading frame. Restriction sites are underlined. Coding sequence is capitalized and start codons indicated in bold text.

[b]The penultimate codon of *mobA*; the stop codon was omitted since a stop codon is provided by the pEGFP-C1 and pEGFP-N1 vectors.

[c]The initiation codon was changed to ATG from GTG to increase the efficiency of translation.

[d]An ATG initiation codon was introduced to allow translation of the 5'-truncated *traI''* and *mobA''* transcripts.

[e]Base 'g' in primer MobR1-human was shifted to 't' in this primer (shown in bold) to avoid binding with the partner primer. The codon designation (R) remains the same.

Template and primer DNA, dNTPs, Expand High Fidelity™ 10x buffer (with $MgCl_2$) and Expand High Fidelity™ Taq Polymerase were mixed in 50µl reaction volumes as per the recommendations of the manufacturer (Roche) with the further addition of 1% dimethyl sulphoxide (DMSO) and 1% dimethyl formamide (DMF) to aid melting of the template DNA (Rybicki, 1996). PCR reactions were carried out in a PTC-150 Minicycler™ (MJ Research). The cycles were as follows: 1 x [94°C, 2 minutes] 10x [94°C, 15 seconds; 55°C (*mobA*) or 47°C (*traI*), 30 seconds; 68°C, 2 minutes] 20x [94°C, 15 seconds; 55°C (*mobA*) or 47°C (*traI*), 30 seconds; 68°C, 2 minutes + a 20 second extension per cycle] 1x [68°C, 7 minutes]. For amplification of the truncated products (650bp-1.4Kb), the elongation time was decreased to 1.5 minutes and the elongation temperature increased to 72°C.

TABLE 1. Primer pairings for creation of TraI and MobA fusions to EGFP[a]

Forward primer	Reverse primer	Vector product	Fusion product
TraF1-B	TraR1-human	pGCF19a and b	Tra-EGFP
TraF1-B	TraR2-human	pGCF20a and b	TraI'-EGFP
TraF1-B-human	TraR1-human	pGCF23a and b	EGFP-TraI
TraF1-B-human	TraR2-human	pGCF24a and b	EGFP-TraI'
MobF1	MobR1-human	pGCF21a and b	MobA-EGFP
MobF1	MobR2-human	pGCF22a and b	MobA'-EGFP
MobF1-human	MobR1-human	pGCF25a and b	EGFP-MobA
MobF1-human	MobR2-human	pGCF26a and b	EGFP-MobA'
TraF2-human	TraR1-human	pGCF27a and b	TraI''-EGFP
MobF2-human	Modified MobR1-human	pGCF28a and b	MobA''-EGFP

[a]Primer positions are mapped on the sequences shown below (Figs 2 and 3).

FIG. 2. *mobA* sequence showing the positions of the forward and reverse primers[a]

```
      1          11         21         31         41         51
      |          |          |          |          |          |
    1 ATGGCGATTT ATCACCTTAC GGCGAAAACC GGCAGCAGGT CGGGCGGCCA ATCGGCCAGG    60
        MobR1/MobR1-human
   61 GCCAAGGCCG ACTACATCCA GCGCGAAGGC AAGTATGCCC GCGACATGGA TGAAGTCTTG   120
  121 CACGCCGAAT CCGGGCACAT GCCGGAGTTC GTCGAGCGGC CCGCCGACTA CTGGGATGCT   180
  181 GCCGACCTGT ATGAACGCGC CAATGGGCGG CTGTTCAAGG AGGTCGAATT TGCCCTGCCG   240
  241 GTCGAGCTGA CCCTCGACCA GCAGAAGGCG CTGGCGTCCG AGTTCGCCCA GCACCTGACC   300
  301 GGTGCCGAGC GCCTGCCGTA TACGCTGGCC ATCCATGCCG GTGGCGGCGA GAACCCGCAC   360
  361 TGCCACCTGA TGATCTCCGA GCGGATCAAT GACGGCATCG AGCGGCCCGC CGCTCAGTGG   420
  421 TTCAAGCGGT ACAACGGCAA GACCCCGGAG AAGGGCGGGG CACAGAAGAC CGAAGCGCTC   480
  481 AAGCCCAAGG CATGGCTTGA GCAGACCCGC GAGGCATGGG CCGACCATGC CAACCGGGCA   540
  541 TTAGAGCGGG CTGGCCACGA CGCCCGCATT GACCACAGAA CACTTGAGGC GCAGGGCATC   600
  601 GAGCGCCTGC CCGGTGTTCA CCTGGGGCCG AACGTGGTGG AGATGGAA GGCCGGGGCATC   660
                                        MobR2-human      MobF2-human
  661 CGCACCGACC GGGCAGACGT GGCCCTGAAC ATCGACACCG CCAACGCCCA GATCATCGAC   720
  721 TTACAGGAAT ACCGGGAGGC AATAGACCAT GAACGCAATC GACAGAGTGA AGAAATCCAG   780
  781 AGGCATCAAC GAGTTAGCGG AGCAGATCGA ACCGCTGGCC CAGAGCATGG CGACACTGGC   840
  841 CGACGAAGCC CGGCAGGTCA TGAGCCAGAC CCAGCAGGCC AGCGAGGCGC AGGCGGCGGA   900
  901 GTGGCTGAAA GCCCAGCGCC AGACAGGGGC GGCATGGGTG GAGCTGGCCA AAGAGTTGCG   960
  961 GGAGGTAGCC GCCGAGGTGA GCAGCGCCGC GCAGAGCGCC CGGAGCGCGT CGCGGGGGTG  1020
 1021 GCACTGGAAG CTATGGCTAA CCGTGATGCT GGCTTCCATG ATGCCTACGG TGGTGCTGCT  1080
 1081 GATCGCATCG TTGCTCTTGC TCGACCTGAC GCCACTGACA ACCGAGGACG GCTCGATCTG  1140
 1141 GCTGCGCTTG GTGGCCCGAT GAAGAACGAC AGGACTTTGC AGGCCATAGG CCGACAGCTC  1200
 1201 AAGGCCATGG GCTGTGAGCG CTTCGATATC GGCGTCAGGG ACGCCACCAC CGGCCAGATG  1260
 1261 ATGAACCGGG AATGGTCAGC CGCCGAAGTG CTCCAGAACA CGCCATGGCT CAAGCGGATG  1320
 1321 AATGCCCAGG GCAATGACGT GTATATCAGG CCCGCCGAGC AGGAGCGGCA TGGTCTGGTG  1380
 1381 CTGGTGGACG ACCTCAGCGA GTTTGACCTG GATGACATGA AAGCCGAGGG CCGGGAGCCT  1440
 1441 GCCCTGGTAG TGGAAACCAG CCCGAAGAAC TATCAGGCAT GGGTCAAGGT GGCCGACGCC  1500
 1501 GCAGGCGGTG AACTTCGGGG GCAGATTGCC CGGACGCTGG CCAGCGAGTA CGACGCCGAC  1560
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1561 CCGGCCAGCG CCGACAGCCG CCACTATGGC CGCTTGGCGG GCTTCACCAA CCGCAAGGAC  1620
1621 AAGCACACCA CCCGCGCCGG TTATCAGCCG TGGGTGCTGC TGCGTGAATC CAAGGGCAAG  1680
1681 ACCGCCACCG CTGGCCCGGC GCTGGTGCAG CAGGCTGGCC AGCAGATCGA GCAGGCCCAG  1740
1741 CGGCAGCAGG AGAAGGCCCG CAGGCTGGCC AGCCTCGAAC TGCCCGAGCG GCAGCTTAGC  1800
1801 CGCCACCGGC GCACGGCGCT GGACGAGTAC CGCAGCGAGA TGGCCGGGCT GGTCAAGCGC  1860
1861 TTCGGTGATG ACCTCAGCAA GTGCGACTTT ATCGCCGCGC AGAAGCTGGC CAGCCGGGGC  1920
1921 CGCAGTGCCG AGGAAATCGG CAAGGCCATG GCCGAGGCCA GCCCAGCGCT GGCAGAGCGC  1980
1981 AAGCCCGGCC ACGAAGCGGA TTACATCGAG CGCACCGTCA GCAAGGTCAT GGGTCTGCCC  2040
2041 AGCGTCCAGC TTGCGCGGGC CGAGCTGGCA CGGGCACCGG CACCCCGCCA GCGAGGCATG  2100
2101 GACAGGGGCG GGCCAGATTT CAGCATGTAG
```

MobR1-human/Modified MobR1-human

[a] *mobA* sequence was obtained from Genbank (accession number NC_001740).

FIG. 3. *traI* sequence (complementary) showing the positions of the forward and reverse primers[a]

```
      1          11         21         31         41         51
      |          |          |          |          |          |
    1 TCATCTACTC CTACCTCGGG TAGTTTTAAG GGAGCCTCGC GGGGTCACGG TGACGGGATC    60
        TraR1-human
   61 ACCGATGGCG AGGCGCTTCA TGCGTTGCAC CGTGGCCTTA TCGACGGGCA GCACCAGAAT   120
  121 CTCGTCGTTT TCTTTCCTCA ACAGGGCCAG CGCCTGGTCC TCGACGTTCC GGGTGCCTGC   180
  181 ATAGGACAGC GCACCAACAT AATCAGTATA TCGTGCATGC TTCGGTATAT CGAAGCCGTT   240
  241 TAGCCGCTTT TGCTCGCGCT CGGCAACATA TTTCTCGGCC GCCGCGATCT GTTCGGGCTT   300
  301 TAGCCCTCTT CCTGGCCCAG AAACTCCCCG TCGCAGTGCG TGAGCTGGTT CGGCTCCTTG   360
  361 CTGCTCCACG TGACCAGGAA CATCACGCGG CAATAGCATT TCAGCTCCGC CGGCGATGCG   420
  421 AACCACACCG AGTTGGGACA GCGCTCGCAA ACGGTTTTGG CTTTGGGGCG GCGGCTTTCG   480
  481 TCCAATGCGT CCAACGTTGG GCTTGCGGAG TGCGACGGTT CCGCCGGCGC TGACGGCGCG   540
  541 AGCGTCCCGT CGGTCGCCGT CGCCGCCTGT GGCGTTGAGG GTGGTTCTGG CTGCGGCAGG   600
  601 TCGAATGCCT CCATCGCCGC CGCGATCTCT TCGTCCGTCA TTTCGTTCGG GTTGCTCATG   660
  661 TGCTTGCTCC TTCGTCAGTA GTTCTTGACG GCGGCGCTCA AGGGCGGCGT CGTCAAAGGT   720
  721 GATTGCCAGA CGGCCAGCGG CGGCCGCCTG CGCGATCCGC TCCTTGAACT CTGCTGTGCC   780
                                                 TraR2-human
  781 CGTTGACGGTGATCCGGTCGC CGAAGCGCTC CATTGCCAGG CGCAGGGCGG CGTCCAGGCC   840
  841 GTCCGTGGTG GCCTCGCGCG AGACTTGCAG GCGGTCGCCG TCGTCGCGGA CGGCGCTGCT   900
  901 GCCGACGCGA TAGATGATGG TTCCCTTCTT CGTGATGTTG TCCGTCACGG CCGCATGGCC   960
  961 CGGCTTGGCC TCGCCGCTGC CCTGGATGGT GTTGCCCTTG AGGTCGCTGC GGCCCTCGCG  1020
 1021 TGCGCGCAGC GCGGCCAGGG CCTTGTCGTC GCCCTTCATC GCCTCGGCCT TGAGCCAGTC  1080
 1081 GGCCCACGCG CGGCGCTGCG TGCGCTCCTG GACCGCCTGA CGGCCCTGCC GGTACTCGCG  1140
 1141 GTTGATCTTG TCCAGGTCGG CGCGCAGAGC CTTGTGCGCC TGCGCGTACA TCAGTCGCTT  1200
 1201 TGCAATGCGC CCCTCGCCCA GCAGCTTGAT AGCGGCGCGG CGCAGCCGGT TGCTGCGCAT  1260
                    TraF2-human
 1261 CGCGGCTTCA ATCAGGCGGT CACGACGCCG GCGCAGCGTG TCCAGCTCGC CCTTGCGCAC  1320
 1321 GGCCCCCATT TCCTGGCGTT CAGACTGATA CCGGGCGTAT AGCTCGGTGG TGTCGATGCG  1380
 1381 GGTCTTGAGC GGCTTCGCTC GATACTCCCG CCGCCGGGGG GCTTCGCCGC CCTCGGCTGG  1440
 1441 CGTGAATGCC CCGAATCGGG CTTCGAGCTT CGGCTTGGAC AGGTCGCGCG AAACGGTGCT  1500
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1501 GGCCTTGACC GTCGTGCCGT CGCCGGCCTC GAAGATGAAG CCGTTTCCGC GCTCGCGCAG  1560
1561 CTTAAGCCCG TTTTCCCGCA GGACGCGGTG CAGGTCCTCC CAGGATTGCG CCGCTTGCAG  1620
1621 CTCCGGCAGG CATTCGCGCT TGATCCAGCC GACCAGGCTT TCCACGCCCG CGTGCCGCTC  1680
1681 CATGTCGTTC GCGCGGTTCT CGGAAACGCG CTGCCGCGTT TCGTGATTGT CACGCTCAAG  1740
1741 CCCGTAGTCC CGTTCGAGCG TCGCGCAGAG GTCAGCGAGG GCGCGGTAGG CCCGATACGG  1800
1801 CTCATGGATG GTGTTTCGGG TCGGGTGAAT CTTGTTGATG GCGATATGGA TGTGCAGGTT  1860
1861 GTCGGTGTCG TGATGCACGG CACTGACGCG CTGATGCTCG GCGAAGCCAA GCCCAGCGCA  1920
1921 GATGCGGTCC TCAATCGCGC GCAACGTCTC CGCGTCGGGC TTCTCTCCCG CGCGGAAGCT  1980
1981 AACCAGCAGG TGATAGGTCT TGTCGGCCTC GGAACGGGTG TTGCCGTGCT GGGTCGCCAT  2040
2041 CACCTCGGCC ATGACAGCGG GCAGGGTGTT TGCCTCGCAG TTCGTGACGC GCACGTGACC  2100
2101 CAGGCGCTCG GTCTTGCCTT GCTCGTCGGT GATGTACTTC ACCAGCTCCG CGAAGTCGCT  2160
2161 CTTCTTGATG GAGCGCATGG GGACGTGCTT GGCAATCAC
                                  TraF1-B/TraF1-B-human
```

[a] *traI* sequence was obtained from Genbank (accession number X54459).

FIG. 4. Predicted amino acid sequence of TraI and MobA fusion proteins[a].

TraI-EGFP

```
     1          11         21         31         41         51
     |          |          |          |          |          |
   1 MIAKHVPMRS IKKSDFAELV KYITDEQGKT ERLGHVRVTN CEANTLPAVM AEVMATQHGN    60
  61 TRSEADKTYH LLVSFRAGEK PDAETLRAIE DRICAGLGFA EHQRVSAVHH DTDNLHIHIA   120
 121 INKIHPTRNT IHEPYRAYRA LADLCATLER DYGLERDNHE TRQRVSENRA NDMERHAGVE   180
 181 SLVGWIKREC LPELQAAQSW EDLHRVLREN GLKLRERGNG FIFEAGDGTT VKASTVSRDL   240
 241 SKPKLEARFG AFTPAEGGEA PRRREYRAKP LKTRIDTTEL YARYQSERQE MGAVRKGELD   300
 301 TLRRRRDRLI EAAMRSNRLR RAAIKLLGEG RIAKRLMYAQ AHKALRADLD KINREYRQGR   360
 361 QAVQERTQRR AWADWLKAEA MKGDDKALAA LRAREGRSDL KGNTIQGSGE AKPGHAAVTD   420
 421 NITKKGTIIY RVGSSAVRDD GDRLQVSREA TTDGLDAALR LAMERFGDRI TVNGTAEFKE   480
 481 RIAQAAAAGR LAITFDDAAL ERRRQELLTK EQAHEQPERN DGRRDRGGDG GIRPAAARTT   540
 541 LNATGGDGDR RDARAVSAGG TVALRKPNVG RIGRKPPPQS QNRLRALSQL GVVRIAGGAE   600
 601 MLLPRDVPGH VEQQGAEPAH ALRRGVSGPG RGLKPEQIAA AEKYVAEREQ KRLNGFDIPK   660
 661 HARYTDYVGA LSYAGTRNVE DQALALLRKE NDEILVLPVD KATVQRMKRL AIGDPVTVTP   720
 721 RGSLKTTRGR SRKDPPVATM VSKGEELFTG VVPILVELDG DVNGHKFSVS GEGEGDATYG   780
 781 KLTLKFICTT GKLPVPWPTL VTTLTYGVQC FSRYPDHMKQ HDFFKSAMPE GYVQERTIFF   840
 841 KDDGNYKTRA EVKFEGDTLV NRIELKGIDF KEDGNILGHK LEYNYNSHNV YIMADKQKNG   900
 901 IKVNFKIRHN IEDGSVQLAD HYQQNTPIGD GPVLLPDNHY LSTQSALSKD PNEKRDHMVL   960
 961 LEFVTAAGIT LGMDELYK
```

EGFP-TraI

```
     1          11         21         31         41         51
     |          |          |          |          |          |
   1 MVSKGEELFT GVVPILVELD GDVNGHKFSV SGEGEGDATY GKLTLKFICT TGKLPVPWPT    60
  61 LVTTLTYGVQ CFSRYPDHMK QHDFFKSAMP EGYVQERTIF FKDDGNYKTR AEVKFEGDTL   120
 121 VNRIELKGID FKEDGNILGH KLEYNYNSHN VYIMADKQKN GIKVNFKIRH NIEDGSVQLA   180
```

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181 DHYQQNTPIG DGPVLLPDNH YLSTQSALSK DPNEKRDHMV LLEFVTAAGI TLGMDELYKS   240
241 GLRSRAQAYM IAKHVPMRSI KKSDFAELVK YITDEQGKTE RLGHVRVTNC EANTLPAVMA   300
301 EVMATQHGNT RSEADKTYHL LVSFRAGEKP DAETLRAIED RICAGLGFAE HQRVSAVHHD   360
361 TDNLHIHIAI NKIHPTRNTI HEPYRAYRAL ADLCATLERD YGLERDNHET RQRVSENRAN   420
421 DMERHAGVES LVGWIKRECL PELQAAQSWE DLHRVLRENG LKLRERGNGF IFEAGDGTTV   480
481 KASTVSRDLS KPKLEARFGA FTPAEGGEAP RRREYRAKPL KTRIDTTELY ARYQSERQEM   540
541 GAVRKGELDT LRRRRDRLIE AAMRSNRLRR AAIKLLGEGR IAKRLMYAQA HKALRADLDK   600
601 INREYRQGRQ AVQERTQRRA WADWLKAEAM KGDDKALAAL RAREGRSDLK GNTIQGSGEA   660
661 KPGHAAVTDN ITKKGTIIYR VGSSAVRDDG DRLQVSREAT TDGLDAALRL AMERFGDRIT   720
721 VNGTAEFKER IAQAAAAGRL AITFDDAALE RRRQELLTKE QAHEQPERND GRRDRGGDGG   780
781 IRPAAARTTL NATGGDGDRR DARAVSAGGT VALRKPNVGR IGRKPPPQSQ NRLRALSQLG   840
841 VVRIAGGAEM LLPRDVPGHV EQQGAEPAHA LRRGVSGPGR GLKPEQIAAA EKYVAEREQK   900
901 RLNGFDIPKH ARYTDYVGAL SYAGTRNVED QALALLRKEN DEILVLPVDK ATVQRMKRLA   960
961 IGDPVTVTPR GSLKTTRGRS RLDPPDLDN
```

Tral'-EGFP

```
      1          11         21         31         41         51
      |          |          |          |          |          |
    1 MIAKHVPMRS IKKSDFAELV KYITDEQGKT ERLGHVRVTN CEANTLPAVM AEVMATQHGN    60
   61 TRSEADKTYH LLVSFRAGEK PDAETLRAIE DRICAGLGFA EHQRVSAVHH DTDNLHIHIA   120
  121 INKIHPTRNT IHEPYRAYRA LADLCATLER DYGLERDNHE TRQRVSENRA NDMERHAGVE   180
  181 SLVGWIKREC LPELQAAQSW EDLHRVLREN GLKLRERGNG FIFEAGDGTT VKASTVSRDL   240
  241 SKPKLEARFG AFTPAEGGEA PRRREYRAKP LKTRIDTTEL YARYQSERQE MGAVRKGELD   300
  301 TLRRRRDRLI EAAMRSNRLR RAAIKLLGEG RIAKRLMYAQ AHKALRADLD KINREYRQGR   360
  361 QAVQERTQRR AWADWLKAEA MKGDDKALAA LRAREGRSDL KGNTIQGSGE AKPGHAAVTD   420
  421 NITKKGTIIY RVGSSAVRDD GDRLQVSREA TTDGLDAALR LAMERFGDRI TVNGTAEFKE   480
  481 KDPPVATMVS KGEELFTGVV PILVELDGDV NGHKFSVSGE GEGDATYGKL TLKFICTTGK   540
  541 LPVPWPTLVT TLTYGVQCFS RYPDHMKQHD FFKSAMPEGY VQERTIFFKD DGNYKTRAEV   600
  601 KFEGDTLVNR IELKGIDFKE DGNILGHKLE YNYNSHNVYI MADKQKNGIK VNFKIRHNIE   660
  661 DGSVQLADHY QQNTPIGDGP VLLPDNHYLS TQSALSKDPN EKRDHMVLLE FVTAAGITLG   720
  721 MDELYK
```

EGFP-TraI'

```
      1          11         21         31         41         51
      |          |          |          |          |          |
    1 MVSKGEELFT GVVPILVELD GDVNGHKFSV SGEGEGDATY GKLTLKFICT TGKLPVPWPT    60
   61 LVTTLTYGVQ CFSRYPDHMK QHDFFKSAMP EGYVQERTIF FKDDGNYKTR AEVKFEGDTL   120
  121 VNRIELKGID FKEDGNILGH KLEYNYNSHN VYIMADKQKN GIKVNFKIRH NIEDGSVQLA   180
  181 DHYQQNTPIG DGPVLLPDNH YLSTQSALSK DPNEKRDHMV LLEFVTAAGI TLGMDELYKS   240
  241 GLRSRAQAYM IAKHVPMRSI KKSDFAELVK YITDEQGKTE RLGHVRVTNC EANTLPAVMA   300
  301 EVMATQHGNT RSEADKTYHL LVSFRAGEKP DAETLRAIED RICAGLGFAE HQRVSAVHHD   360
  361 TDNLHIHIAI NKIHPTRNTI HEPYRAYRAL ADLCATLERD YGLERDNHET RQRVSENRAN   420
  421 DMERHAGVES LVGWIKRECL PELQAAQSWE DLHRVLRENG LKLRERGNGF IFEAGDGTTV   480
  481 KASTVSRDLS KPKLEARFGA FTPAEGGEAP RRREYRAKPL KTRIDTTELY ARYQSERQEM   540
  541 GAVRKGELDT LRRRRDRLIE AAMRSNRLRR AAIKLLGEGR IAKRLMYAQA HKALRADLDK   600
  601 INREYRQGRQ AVQERTQRRA WADWLKAEAM KGDDKALAAL RAREGRSDLK GNTIQGSGEA   660
  661 KPGHAAVTDN ITKKGTIIYR VGSSAVRDDG DRLQVSREAT TDGLDAALRL AMERFGDRIT   720
  721 VNGTAEFKEL DPPDLDN
```

MobA-EGFP

```
      1          11         21         31         41         51
      |          |          |          |          |          |
    1 MAIYHLTAKT GSRSGGQSAR AKADYIQREG KYARDMDEVL HAESGHMPEF VERPADYWDA    60
   61 ADLYERANGR LFKEVEFALP VELTLDQQKA LASEFAQHLT GAERLPYTLA IHAGGGENPH   120
  121 CHLMISERIN DGIERPAAQW FKRYNGKTPE KGGAQKTEAL KPKAWLEQTR EAWADHANRA   180
  181 LERAGHDARI DHRTLEAQGI ERLPGVHLGP NVVEMEGRGI RTDRADVALN IDTANAQIID   240
  241 LQEYREAIDH ERNRQSEEIQ RHQRVSGADR TAGPEHGDTG RRSPAGHEPD PAGQRGAGGG   300
  301 VAESPAPDRG GMGGAGQRVA GGSRRGEQRR AERPERVAGV ALEAMANRDA GFHDAYGGAA   360
  361 DRIVALARPD ATDNRGRLDL AALGGPMKND RTLQAIGRQL KAMGCERFDI GVRDATTGQM   420
  421 MNREWSAAEV LQNTPWLKRM NAQGNDVYIR PAEQERHGLV LVDDLSEFDL DDMKAEGREP   480
  481 ALVVETSPKN YQAWVKVADA AGGELRGQIA RTLASEYDAD PASADSRHYG RLAGFTNRKD   540
  541 KHTTRAGYQP WVLLRESKGK TATAGPALVQ QAGQQIEQAQ RQQEKARRLA SLELPERQLS   600
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601 RHRRTALDEY RSEMAGLVKR FGDDLSKCDF IAAQKLASRG RSAEEIGKAM AEASPALAER   660
661 KPGHEADYIE RTVSKVMGLP SVQLARAELA RAPAPRQRGM DRGGPDFSMR QDPPVATMVS   720
721 KGEELFTGVV PILVELDGDV NGHKFSVSGE GEGDATYGKL TLKFICTTGK LPVPWPTLVT   780
781 TLTYGVQCFS RYPDHMKQHD FFKSAMPEGY VQERTIFFKD DGNYKTRAEV KFEGDTLVNR   840
841 IELKGIDFKE DGNILGHKLE YNYNSHNVYI MADKQKNGIK VNFKIRHNIE DGSVQLADHY   900
901 QQNTPIGDGP VLLPDNHYLS TQSALSKDPN EKRDHMVLLE FVTAAGITLG MDELYK
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EGFP-MobA

```
    1          11         21         31         41         51
    |          |          |          |          |          |
  1 MVSKGEELFT GVVPILVELD GDVNGHKFSV SGEGEGDATY GKLTLKFICT TGKLPVPWPT    60
 61 LVTTLTYGVQ CFSRYPDHMK QHDFFKSAMP EGYVQERTIF FKDDGNYKTR AEVKFEGDTL   120
121 VNRIELKGID FKEDGNILGH KLEYNYNSHN VYIMADKQKN GIKVNFKIRH NIEDGSVQLA   180
181 DHYQQNTPIG DGPVLLPDNH YLSTQSALSK DPNEKRDHMV LLEFVTAAGI TLGMDELYKS   240
241 GLRSRAQAWM AIYHLTAKTG SRSGGQSARA KADYIQREGK YARDMDEVLH AESGHMPEFV   300
301 ERPADYWDAA DLYERANGRL FKEVEFALPV ELTLDQQKAL ASEFAQHLTG AERLPYTLAI   360
361 HAGGGENPHC HLMISERIND GIERPAAQWF KRYNGKTPEK GGAQKTEALK PKAWLEQTRE   420
421 AWADHANRAL ERAGHDARID HRTLEAQGIE RLPGVHLGPN VVEMEGRGIR TDRADVALNI   480
481 DTANAQIIDL QEYREAIDHE RNRQSEEIQR HQRVSGADRT AGPEHGDTGR RSPAGHEPDP   540
541 AGQRGAGGGV AESPAPDRGG MGGAGQRVAG GSRRGEQRRA ERPERVAGVA LEAMANRDAG   600
601 FHDAYGGAAD RIVALARPDA TDNRGRLDLA ALGGPMKNDR TLQAIGRQLK AMGCERFDIG   660
661 VRDATTGQMM NREWSAAEVL QNTPWLKRMN AQGNDVYIRP AEQERHGLVL VDDLSEFDLD   720
721 DMKAEGREPA LVVETSPKNY QAWVKVADAA GGELRGQIAR TLASEYDADP ASADSRHYGR   780
781 LAGFTNRKDK HTTRAGYQPW VLLRESKGKT ATAGPALVQQ AGQQIEQAQR QQEKARRLAS   840
841 LELPERQLSR HRRTALDEYR SEMAGLVKRF GDDLSKCDFI AAQKLASRGR SAEEIGKAMA   900
901 EASPALAERK PGHEADYIER TVSKVMGLPS VQLARAELAR APAPRQRGMD RGGPDFSMRQ   960
961 DPPDLDN
```

MobA'-EGFP

```
      1          11         21         31         41         51
      |          |          |          |          |          |
    1 MAIYHLTAKT GSRSGGQSAR AKADYIQREG KYARDMDEVL HAESGHMPEF VERPADYWDA    60
   61 ADLYERANGR LFKEVEFALP VELTLDQQKA LASEFAQHLT GAERLPYTLA IHAGGGENPH   120
  121 CHLMISERIN DGIERPAAQW FKRYNGKTPE KGGAQKTEAL KPKAWLEQTR EAWADHANRA   180
  181 LERAGHDARI DHRTLEAQGI ERLPGVHLGP NVVEMEREDP PVATMVSKGE ELFTGVVPIL   240
  241 VELDGDVNGH KFSVSGEGEG DATYGKLTLK FICTTGKLPV PWPTLVTTLT YGVQCFSRYP   300
  301 DHMKQHDFFK SAMPEGYVQE RTIFFKDDGN YKTRAEVKFE GDTLVNRIEL KGIDFKEDGN   360
  361 ILGHKLEYNY NSHNVYIMAD KQKNGIKVNF KIRHNIEDGS VQLADHYQQN TPIGDGPVLL   420
  421 PDNHYLSTQS ALSKDPNEKR DHMVLLEFVT AAGITLGMDE LYK
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EGFP-MobA'

```
      1          11         21         31         41         51
      |          |          |          |          |          |
    1 MVSKGEELFT GVVPILVELD GDVNGHKFSV SGEGEGDATY GKLTLKFICT TGKLPVPWPT    60
   61 LVTTLTYGVQ CFSRYPDHMK QHDFFKSAMP EGYVQERTIF FKDDGNYKTR AEVKFEGDTL   120
  121 VNRIELKGID FKEDGNILGH KLEYNYNSHN VYIMADKQKN GIKVNFKIRH NIEDGSVQLA   180
  181 DHYQQNTPIG DGPVLLPDNH YLSTQSALSK DPNEKRDHMV LLEFVTAAGI TLGMDELYKS   240
  241 GLRSRAQAWM AIYHLTAKTG SRSGGQSARA KADYIQREGK YARDMDEVLH AESGHMPEFV   300
  301 ERPADYWDAA DLYERANGRL FKEVEFALPV ELTLDQQKAL ASEFAQHLTG AERLPYTLAI   360
  361 HAGGGENPHC HLMISERIND GIERPAAQWF KRYNGKTPEK GGAQKTEALK PKAWLEQTRE   420
  421 AWADHANRAL ERAGHDARID HRTLEAQGIE RLPGVHLGPN VVEMEREDPP DLDN
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TraI''-EGFP

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      1          11         21         31         41         51
      |          |          |          |          |          |
    1 MAIKLLGEGR IAKRLMYAQA HKALRADLDK INREYRQGRQ AVQERTQRRA WADWLKAEAM    60
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  61 KGDDKALAAL RAREGRSDLK GNTIQGSGEA KPGHAAVTDN ITKKGTIIYR VGSSAVRDDG   120
 121 DRLQVSREAT TDGLDAALRL AMERFGDRIT VNGTAEFKER IAQAAAAGRL AITFDDAALE   180
 181 RRRQELLTKE QAHEQPERND GRRDRGGDGG IRPAAARTTL NATGGDGDRR DARAVSAGGT   240
 241 VALRKPNVGR IGRKPPPQSQ NRLRALSQLG VVRIAGGAEM LLPRDVPGHV EQQGAEPAHA   300
 301 LRRGVSGPGR GLKPEQIAAA EKYVAEREQK RLNGFDIPKH ARYTDYVGAL SYAGTRNVED   360
 361 QALALLRKEN DEILVLPVDK ATVQRMKRLA IGDPVTVTPR GSLKTTRGRS RKDPPVATMV   420
 421 SKGEELFTGV VPILVELDGD VNGHKFSVSG EGEGDATYGK LTLKFICTTG KLPVPWPTLV   480
 481 TTLTYGVQCF SRYPDHMKQH DFFKSAMPEG YVQERTIFFK DDGNYKTRAE VKFEGDTLVN   540
 541 RIELKGIDFK EDGNILGHKL EYNYNSHNVY IMADKQKNGI KVNFKIRHNI EDGSVQLADH   600
 601 YQQNTPIGDG PVLLPDNHYL STQSALSKDP NEKRDHMVLL EFVTAAGITL GMDELYK
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MobA''-EGFP

```
     1          11         21         31         41         51
     |          |          |          |          |          |
   1 MGRGIRTDRA DVALNIDTAN AQIIDLQEYR EAIDHERNRQ SEEIQRHQRV SGADRTAGPE    60
  61 HGDTGRRSPA GHEPDPAGQR GAGGGVAESP APDRGGMGGA GQRVAGGSRR GEQRRAERPE   120
 121 RVAGVALEAM ANRDAGFHDA YGGAADRIVA LARPDATDNR GRLDLAALGG PMKNDRTLQA   180
 181 IGRQLKAMGC ERFDIGVRDA TTGQMMNREW SAAEVLQNTP WLKRMNAQGN DVYIRPAEQE   240
 241 RHGLVLVDDL SEFDLDDMKA EGREPALVVE TSPKNYQAWV KVADAAGGEL RGQIARTLAS   300
 301 EYDADPASAD SRHYGRLAGF TNRKDKHTTR AGYQPWVLLR ESKGKTATAG PALVQQAGQQ   360
 361 IEQAQRQQEK ARRLASLELP ERQLSRHRRT ALDEYRSEMA GLVKRFGDDL SKCDFIAAQK   420
 421 LASRGRSAEE IGKAMAEASP ALAERKPGHE ADYIERTVSK VMGLPSVQLA RAELARAPAP   480
 481 RQRGMDRGGP DFSMDRGGPD FSMRQDPPVA TMVSKGEELF TGVVPILVEL DGDVNGHKFS   540
 541 VSGEGEGDAT YGKLTLKFIC TTGKLPVPWP TLVTTLTYGV QCFSRYPDHM KQHDFFKSAM   600
 601 PEGYVQERTI FFKDDGNYKT RAEVKFEGDT LVNRIELKGI DFKEDGNILG HKLEYNYNSH   660
 661 NVYIMADKQK NGIKVNFKIR HNIEDGSVQL ADHYQQNTPI GDGPVLLPDN HYLSTQSALS   720
 721 KDPNEKRDHM VLLEFVTAAG ITLGMDELYK
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[a]EGFP sequence is italisized, amino acids from the multicloning site are underlined. Protein sequences were obtained from genbank (accession numbers NP_044304 and CAA38336).

TABLE 2. Predicted Characteristics of the Putative Conjugative Export Proteins[a]

protein	predicted size (kDa)	predicted pI	% basic amino acids	putative NLS?[d]	predicted subcellular location
TraI	82 [109][b]	9.98 [9.43][b]	18.3% (13% R, 5.3% K)[c]	✓/X	nucleus (0.960)[e] [nucleus]
TraI'	54 [82]	9.62 [8.84]	18.3% (12.1% R, 5.8% K)	✓/X	nucleus (0.960) [nucleus]
TraI''	45 [72]	10.41 [9.44]	18.7% (13.1% R, 5.6% K)	✓/X	cytoplasm[f] (0.450) [cytoplasm]
MobA	78 [106]	6.76 [6.3]	14.7% (10.7% R, 3.9% K)	X/X	nucleus[f] (0.300) [nucleus]
MobA'	24 [52]	6.01 [5.75]	11.9% (7.9% R, 5.1% K)	X/X	cytoplasm (0.450) [cytoplasm]
MobA''	55 [83]	8.41 [6.39]	15.3% (11.9% R, 3.4% K)	X/X	MMS[f,g] (0.360) [MMS][f]
VirD1	16	8.93	13.6% (10.9% R, 2.7% K)	X/X	MMS (0.477)
VirD2	48	7.97	15.8% (11.6% R, 4.2% K)	✓/✓	nucleus (0.880)
VirE2	61	6.57	13.6% (8.3% R, 5.3% K)	X/X	cytoplasm (0.685)
VIP-1	38	7.13	12.9% (7.3% R, 5.6% K0	✓/X	nucleus (0.760)

[a] Protein characteristics were predicted by the computer programs PSORT (http://psort.nibb.ac.jp), PSORT II (http://psort.nibb.ac.jp/form2.html), ExPASy (http://www.expasy.ch/tools/protparam.html) and PredictNLS (http://cubic.bioc.columbia.edu).

[b] The data in brackets represents fusion proteins with EGFP on the C terminus.

[c] R = arginine, K = lysine.

[d] PSORT/PredictNLS. PSORT predicts subcellular location by running a series of tests. Nuclear localization is assigned based on the presence of NLS sequences and the proportion of basic amino acid residues. PredictNLS finds nuclear localization sequences within submitted protein sequences by searching a database of known nuclear localization sequences.

[e] Figures in parentheses represent the likelihood of the predicted protein subcellular location being correct (PSORT)

[f] Where the likelihood scores were low, different subcellular locations were designated depending on which version of PSORT was used. The stated locations were predicted by PSORT. Alternatively, PSORT II predicted TraI', MobA, MobA'' and MobA''-EGFP to be nuclear, mitochondrial, nuclear and cytoplasmic, respectively.

[g] Mitochondrial matrix space.

LITERATURE CITED

Rybicki, E. (1996) PCR primer design and reaction optimization. In *Molecular Biology Techniques Manual*. Coyne, V.E., James, M.D., Reid, S.J. and Rybicki, E.P. (eds.): Molecular Biology Department, University of Cape Town.